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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

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 US Department of Commerce
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 in its capacity as elected Office

Date of mailing (day/month/year) 31 October 2000 (31.10.00)	
International application No. PCT/US00/05594	Applicant's or agent's file reference 4995.52-304
International filing date (day/month/year) 03 March 2000 (03.03.00)	Priority date (day/month/year) 03 March 1999 (03.03.99)
Applicant LEWANDOWSKI, Gail et al	

1. The designated Office is hereby notified of its election made:



in the demand filed with the International Preliminary Examining Authority on:

03 October 2000 (03.10.00)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Diana Nissen
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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Applicant's or agent's file reference 4995.52-304		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/05594	International filing date (day/month/year) 03/03/2000	Priority date (day/month/year) 03/03/1999	
International Patent Classification (IPC) or national classification and IPC C12N15/86			
Applicant LEWANDOWSKI, Gail et al.			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 8 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"> I <input checked="" type="checkbox"/> Basis of the report II <input type="checkbox"/> Priority III <input checked="" type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability IV <input type="checkbox"/> Lack of unity of invention V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement VI <input type="checkbox"/> Certain documents cited VII <input type="checkbox"/> Certain defects in the international application VIII <input checked="" type="checkbox"/> Certain observations on the international application 			
Date of submission of the demand 03/10/2000		Date of completion of this report 12.04.2001	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer Paresce, D Telephone No. +49 89 2399 8995	



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/05594

1. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):
Description, pages:

1-34 as originally filed

Claims, No.:

1-87 as originally filed

Drawings, sheets:

1/12-12/12 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
☐ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

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- ☐ the drawings, sheets:
5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):
(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:
- ☐ the entire international application.
- ☒ claims Nos. 11-48, 62-65, 82-87.

because:

- ☒ the said international application, or the said claims Nos. 11-48, 62-65, 82-87 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .
2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 8-10, 15-16, 29-31, 36, 38-40, 42-44, 46-48, 50-51, 59-65, 70-75,

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	No:	Claims	80-81, 83, 85-87
			1-7, 11-14, 17-28, 32-35, 37, 41, 45, 49, 52-58, 66-69, 76-79, 82, 84
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-87
Industrial applicability (IA)	Yes:	Claims	1-10, 49-61, 66-81
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

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Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 11-48, 62-65, 82-87 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present claims 11-48, 62-65, 82-87 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

- 1) The documents mentioned in this communication are numbered as in the search report, i.e. D1 corresponds to the first document of the search report.
- 2) Novelty: Article 33(2) PCT

The subject-matter of claims 1-7, 11-14, 17-28, 32-35, 37, 41, 45, 49, 52-58, 66-69, 76-79, 82, 84 is not considered new in the sense of Article 33(2) PCT for the following reasons:

D1 describes the cloning of the mouse IFN- γ coding sequence into a HSV-based amplicon vector (HSV(p IFN- γ)). The HSV based amplicon was selected because HSV-packaged vectors are transynaptically transported. Thus by using the same inoculation route for HSV-2 and HSV(p IFN- γ), IFN- γ production occurs exactly in

HSV-2 infected nervous system regions (see D1, p. 67, left column). The construction of HSV(p IFN- γ) is shown in figure 1 and on p. 68 of D1. In brief, the HSV(p IFN- γ) comprises an 'a' region for HSV-1 DNA packaging into virions, an HSV-1 origin of DNA sequence, a transcriptional unit containing an HSV-1 immediate early gene promoter, and the ampicillin resistance conferring sequence (see figure 1). D1 discloses the development of an HSV-based amplicon vector producing mouse IFN- γ and describes the use of said vector for treating mice infected with a virulent HSV-2 strain. Mice that were co-inoculated with HSV-2(G) and HSV(p IFN- γ) survived infection. Cessation of HSV replication closely followed the 10-fold increase intracerebral IFN- γ . It is suggested, therefore, that a deficient IFN- γ response is a basis for HSV-2 neurovirulence in mice (see p. 73). Furthermore, it is mentioned in D1 that the use of HSV(p IFN- γ) to protect HSV-2 infected mice from death suggests a potential gene-therapy approach for HSV encephalitis (see p. 74).

The IPEA is of the opinion that the methods and compositions disclosed in D1 would fall under the scope of claims 1-7, 11-14, 17-28, 32-35, 37, 41, 45, 49, 52-58, 66-69, 76-79, 82, 84 of the present application. These claims are vague and contain no specific technical features which would make it possible to distinguish the claimed methods from those disclosed in D1 (see also paragraph VIII below).

D2 describes methods of testing a herpes simplex virus amplicon vector for its ability to transduce murine epidermis through topical application. The HSV-1 amplicon viral vector containing the lacZ reporter gene was found to transduce murine epidermis with high efficiency (see p. 803).

D3 describes a defective HSV-1 vector comprising the HSV-1 immediate-early promoter to regulate expression of the *E. coli* lacZ gene, an HSV-1 origin of replication and a packaging site. These HSV-1 vectors, in the presence of helper virus, can undergo DNA replication and packaging into HSV-1 particles. The HSV-1 vector was delivered into the adult rat striatum or hippocampus and 2-3 months after gene transfer, superinfection with several replication-incompetent HSV-1 mutants, resulted in reactivation of the vector gene expression (see p. 615-616). Claims 1-6, 11-14 are not considered novel in view of D3. These claims are so vague and broad in scope that the IPEA is of the opinion that the information

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provided in D3 would fall under the scope of these claims.

The subject-matter of claims 8-10, 15-16, 29-31, 36, 38-40, 42-44, 46-48, 50-51, 59-65, 70-75, 80-81, 83, 85-87 has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

2) Inventive Step: Article 33(3) PCT

The subject-matter of claims 1-87 is not considered to involve an inventive step in the sense of Article 33(3) PCT for the following reasons:

D1 is regarded as being the closest prior art to the subject-matter of these claims.

D1 describes the treatment of HSV-2 infected mice with an HSV-based amplicon vector expressing mouse IFN- γ . The HSV-based amplicon vector described in D1 was constructed using an HSV-1 helper virus. It is mentioned in D1 that HSV-1(F) was used as the helper virus because (i) "p IFN- γ could not be packaged by HSV-2 strains, (ii) HSV-1(F) has the same neurotropism as HSV-2 and is avirulent in mice" (p.69-70).

It is mentioned on p. 18 of the present application that although the increased survival of HSV-1(p IFN- γ)-treated animals suggests that the amplicon may have been packaged *in situ* by the actively replicating HSV-2 virus, *in vitro* studies (not shown) indicate that HSV-2 virions inefficiently package and replicate amplicon based on HSV-1 DNA. This could be due to the mismatch of HSV-2 virions with HSV-1 a' and/or HSV-1 ori sequences. Therefore, the present application discloses both HSV-1 and HSV-2-based amplicon vectors. The IPEA, however, considers that methods or compositions based on the use of HSV-2-based amplicon vectors rather than the HSV-1 based amplicon vectors described in D1 do not involve an inventive step.

The procedures for constructing HSV-2-based amplicon vectors described in the present application are employed in D1, for the same purposes as those of the present application, using HSV-1(F) as the helper virus. It would be obvious to the person skilled in the art, namely when the same result is to be achieved, to apply

the methods according to D1 with corresponding effect to an HSV-2 based vector. The construction of an HSV-2 based amplicon vector using the teachings of D1 is considered to come within the scope of the customary practice followed by persons skilled in the art. The HSV-2 based amplicon vector and methods involving the use of said vector as claimed in the present application can only be regarded as inventive, if said vector or methods presented unexpected effects or properties in relation to those disclosed in D1. However, no such effects or properties are indicated in the application. Therefore, an inventive step for the claimed methods or compositions cannot at present be recognized, unless said methods will show some kind of unexpected advantages over those described in prior art, which should be demonstrated.

VIII. Certain observations on the international application

1) Clarity: Article 6 PCT

Article 6 PCT requires amongst other things that the claims, which define the matter for which protection is sought (i.e. the object of invention) be clear. This has to be interpreted as meaning not only that a claim from a technical point of view must be comprehensible, but also that it must define clearly the object of the invention, that is to say, it must indicate all the essential features thereof. The essential features are regarded as all features which are necessary to obtain the desired effect, or differently expressed, those features which are necessary to solve the technical problem with which the application is concerned. In other words, all technical features which enable the skilled person to put the claimed matter into practice without undue burden i.e. without experimentation or without application of inventive skill.

Claims 1, 11, 22, 49, 62, 66, 82 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result should be added.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁷ : C12N 15/86, A61K 48/00, 38/21, 47/20, 47/30, A61P 31/22</p>	<p>A1</p>	<p>(11) International Publication Number: WO 00/52185 (43) International Publication Date: 8 September 2000 (08.09.00)</p>
<p>(21) International Application Number: PCT/US00/05594 (22) International Filing Date: 3 March 2000 (03.03.00) (30) Priority Data: 60/123,071 3 March 1999 (03.03.99) US (71)(72) Applicants and Inventors: LEWANDOWSKI, Gail [US/US]; 5800 North Kolb, Apt. 12266, Tuscon, AZ 85750 (US). PRINCE, Gregory, A. [US/US]; 14800 Petit Way, Potomac, MD (US). GUZOWSKI, John [US/US]; 5800 North Kolb, Tuscon, AZ 85750 (US). (74) Agents: GARRETT, Arthur, S. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street, N.W., Washington, DC 20005-3315 (US).</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: APPLICATION OF DNA VECTORS FOR THE TREATMENT OF VIRAL INFECTION (57) Abstract The present invention relates to DNA-based constructs and methods for treating viral infections. In a preferred embodiment, an HSV based amplicon is topically applied to the site of an actively replicating HSV infection, initiating a four-tier anti-viral therapy. First, IFN-γ expression from a constitutive promoter exerts an antiviral effect at the site of active infection. Second, replication machinery of the resident virus packages the amplicon into HSV virions, <i>in situ</i>, thereby decreasing the infectious titer. Third, HSV virion containing amplicon DNA are transported into the nervous system where IFN-γ expression promotes the establishment of latency. Fourth, as the host is exposed to emotional or physical stressor that reactivate the virus, amplicon-directed IFN-γ is expressed. Increasing titers of IFN-γ enforce viral latency.</p>		

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Application of DNA Vectors for the Treatment of Viral Infection

GOVERNMENT INTEREST

This invention was made, in part, with Government support under NIH grant no. RO1-MH58374 awarded by the National Institutes of Health. The U.S. Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to DNA-based constructs and methods for treating viral infections. The preferred constructs are amplicons containing DNA that can be packaged *in situ* by an active viral infection, preferably a Herpesvirus infection, and more preferably, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) infections. The amplicons may express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). In a highly preferred embodiment, one or more amplicons express IFN- γ in the epithelial and neural cells of an organism infected with HSV.

The present invention further relates to the use of these constructs for the treatment of viral infections. In a highly preferred embodiment, amplicon DNA is applied topically to a site of active HSV infection and packaged *in situ*. Locally, amplicon-directed cytokine expression reduces the titer of HSV to ameliorate the severity and duration of the active (or productive) infection. Amplicon DNA packaged *in situ* by active HSV is transported to the nervous system. In the nervous system amplicon-directed IFN- γ expression induces the virus to enter a latent phase. The amplicons of the invention are constructed so that environmental stimuli which reactivate HSV also induce the amplicon-directed expression of IFN- γ . The induced IFN- γ maintains or re-establishes HSV latency, thereby eliminating or reducing recurrent infection.

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BACKGROUND

The human Herpesviruses comprise at least eight distinct DNA viruses, including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and three recently described viruses designated human herpesvirus types 6, 7, and 8. In addition, a large number of Herpesviruses cause disease in non-human hosts including, Equid herpesviruses 1-8; Suid herpesviruses 1 and 2; and Gallid herpes viruses 1-3. Known members of the Herpesviridae family are reviewed in Bernard Roizman, Herpesviridae, Fields Virology, 3d ed., Ch. 71, incorporated herein by reference. All herpes viruses produce both active and latent infections, which permits viral reactivation and recurrent clinical illness throughout the life of the host. The recurrent nature of Herpesvirus infection promotes transmission and contributes to the high prevalence of these viruses.

HSV-1 and HSV-2 (collectively, HSV) have been identified as the causative agents of recurrent oral and genital herpetic lesions. HSV-1 and HSV-2 are highly related and present clinically indistinguishable symptoms. Nevertheless, each exhibits preferential sites of infection such that HSV-1 is primarily responsible for oral-labialis herpes, whereas HSV-2, and to a lesser extent, HSV-1, are responsible for genital and anal herpes.

Clinical manifestations of HSV infection are reviewed in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d ed., Ch. 73, incorporated herein by reference. HSV possesses both a lytic and a latent phase. The lytic phase begins with exposure to HSV at mucosal surfaces or abraded skin. The virus gains entry into epidermal and dermal cells and initiates viral replication.

The active HSV infections of the lytic cycle frequently result in painful vesicles filled with highly infectious fluid appearing on the dermal and mucosal surfaces of the lips, mouth, pharynx, and nasal passages (oral-labial herpes), and genitals, buttocks, thighs and anal regions (genital herpes). Vesicle formation is frequently preceded by prodrome stage, characterized by a tingling or burning sensation. The infection site reddens (macule or erythema stage) and fluid-filled vesicles appear rapidly after the onset of prodrome, within about six hours in the case HSV-1 infection. Within 3 to 4

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days the vesicular fluid becomes pustular due the recruitment of inflammatory cells. On dry, external surfaces, the lesions develop a crusting, scab-like appearance, whereas shallow ulcers are more common on moist mucosal membranes. Untreated, oral infections are generally resolved in 8-10 days, but genital lesions persist for an average of three weeks.

Following primary infection, the virus travels to the neuronal nuclei in the dorsal root ganglia of the sacral, vagal, and/or trigeminal nerves where it remains latent for indefinite periods. While in a latent state, HSV does not produce infectious virus, does not cause disease, and is not transmissible. However, when HSV reactivates, it is transmissible, even if the episode is subclinical or asymptomatic. (Barton et al., *Int. J. STD. AIDS* 7:229-32 (1996)). Reactivation may be triggered by emotional stress and a variety of environmental factors including, UV light, trauma, heat, fever, colds, fatigue, menses, and certain foods, and usually results in the appearance of new lesions at or near the site of initial infection.

The frequency of reactivation is patient-dependent but may be quite high. About 23% of those infected orally with HSV-1 experience 2 or more recurrences per year. Genital (primarily HSV-2) outbreaks follow a somewhat different pattern. About 500,000 to one million new cases of genital herpes occur in the United States alone. Although only 20-25% of those infected with genital herpes are aware of their condition, more than half of the patients who experience recurrences report at least one episode per month. (Hamuy, R. and B. Berman. 1998. Treatment of Herpes simplex virus infections with topical antiviral agents. *Eur. J. Dermatol.* 8:310-319).

HSV-1 and HSV-2 are easily transmissible and a large proportion of the population has been infected. Worldwide, 60 to 90% of adults have been infected with HSV-1, and nearly 25% have been infected with HSV-2. HSV is not commonly considered a critical health concern among immunocompetent adults, but for people who suffer frequent oral or genital recurrences, herpes can greatly decrease the quality of life. Although frequently painful, lesions generally heal completely. However, severe or recurrent outbreaks can result in scarring, and infections of the eye may result in corneal blindness. (John Manos, DNA Viruses 1: Herpesviruses, Microbiology and infectious

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diseases). Damage to peripheral nerves may result in facial paralysis or Bell's palsy, and movement disorders in children. (Billue, Nurse. Pract. 22:88, 97-100, and 102-105 (1997); Hargrave et al., Dev. Med. Child. Neurol. 40:640-42 (1998)).

In immunocompromised patients, including patients receiving immunosuppressant drugs in association with organ or bone marrow transplants and those with the acquired immunodeficiency syndrome (AIDS), lesions may be chronic, progressive and disabling. Similarly, immunological changes occurring during pregnancy place both the mother and fetus at a high risk of severe infection. Active infection during pregnancy has been associated with spontaneous abortion and occasionally with life-threatening systemic maternal infection. (Fields, Bernard, et al., Fields Virology, Herpes simplex viruses, vol. 2, Ch. 73).

Although the spread of HSV through the nervous system is principally confined to peripheral nerves, HSV is implicated in a particularly lethal form of viral encephalitis. HSV encephalitis is the most common viral infection of the central nervous system, accounting for 10-20% of all isolated viral CNS infections in the United States. Left untreated, HSV encephalitis has a mortality rate of about 70%. Less well recognized CNS associations include mood and behavioral dysfunctions, Alzheimer's disease, and profound global amnesia. (Becker, Y., Virus Genes 10:217-226 (1995); Caparros-Lefebvre, D., et al, J. Neurol. 243:248-256 (1996); Itzhaki, R. F., et al., Lancet 352:238 (1998); Lin et al., Mol. Chem. Neuropathol. 28:135-41 (1996); Kimura, S., et al., Intern. Med. 34:131-133 (1995); Wilson, B. A., et al., J. Clin. Exp. Neuropsychol. 17:668-681 (1995)).

In accord with these CNS disturbances, studies in mice and cotton rats strongly argue that HSV invades the brain during primary ocular HSV infection (Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994) and Lewandowski et al., Proc. Natl. Acad. Sci. USA 90:2005-2009 (1993)), and primary HSV labialis infection (Lewandowski et al. manuscript in preparation). Moreover, because HSV moves along the nerves in both retrograde and anterograde directions, HSV has the potential to spread further into the nervous system with each recurrent episode. Consequently, even if initially limited to the peripheral nervous system, HSV has the potential of invading the brain every time

latency is broken. Notably, no current herpes therapy is designed to keep HSV in a latent state.

Currently, nucleoside analogs have proven somewhat successful at inhibiting HSV replication in the active stages of infection. These compounds include competitive inhibitors of HSV DNA polymerase: the pyrophosphate, Foscarnet, and the acyclic nucleoside phosphonate, cidofovir (De Clercq, E., *Intervirology* 40:295-303 (1997); Martinez, C. M. et al., *Pharmacotherapy* 31:1519-1521 (1997); Oram, R. J., et al, *Pediatr. Infect. Dis. J.* 17:652-653 (1998)). However, nearly all current herpes therapies rely on acyclic nucleoside analogs such as acyclovir (ACV), valacyclovir, famciclovir, penciclovir (PCV), and ganciclovir (Erlich, K., et al., *J. Med.* 166:211-215 (1997); Oram, R. J., et al., *Pediatr. Infect. Dis. J.* 17:652-653 (1998); Wutzler, P., *Intervirology* 40:343-356 (1997)). These compounds are selectively monophosphorylated by the herpesvirus thymidine kinase, but not by the cellular enzyme. The monophosphate nucleotide analog is then further phosphorylated by cellular kinases to the di- and triphosphate forms. The triphosphorylated nucleotide analog interacts with and inhibits the viral DNA polymerase.

In some patients, oral or intravenous administration of nucleoside analog compounds result in a faster healing of lesions, a shortened duration of virus shedding, and a faster resolution of symptoms. In addition, suppressive (daily) oral acyclovir, valacyclovir, or famciclovir may reduce the severity and frequency of recurrent episodes in some patients (Erlich, K. S., *West. J. Med.* 166:211-215 (1997); Faro, S., *Infect. Dis. Obstet. Gynecol.* 6:38-43, (1998)). However, it is likely that suppressive dosage schedules will increase the inherent risk of drug-resistant HSV strains (Reusser, P., *J. Hosp. Infect.* 33:235-248 (1996)). Indeed, acyclovir-resistant and Foscarnet-resistant HSVs have already been described. (Erlich, K. S., *West. J. Med.* 166:211-215 (1997)).

HSV infection can also be treated by topical administration of anti-herpetic drugs. (Hamuy, R. et al., *Eur. J. Dermatol.* 8:310-319 (1998)). However, topical therapies have at least two challenges. First, the drug must be specially formulated to penetrate the stratum corneum barrier in the skin. Currently, the approved topical formulations include ACV in polyethylene glycol, and ACV or PCV in an aqueous cream. Unfortunately, transdermal delivery in these approved vehicles is very poor and slow. (Freeman, D.

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J., et al., Antimicrob. Agents. Chemother. 29:730-732; Spruance, S. L., J. Infect. Dis. 146:85-90 (1982)). Secondly, topical application must begin during the brief "prodrome" stage, concurrent with the initiation of HSV replication. Nevertheless, patient-initiated applications frequently do not begin until the lesion becomes visible and the HSV titer is already high.

Consequently, there remains a need in the art for a safe and efficacious treatment for HSV. In particular, there remains a need for treatment which will prevent recurrent outbreaks of active infection, reduce viral transmission, and limit the propagation of infected tissues in a host.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing a novel system for treating viral infection, preferably, Herpesvirus infections, and more preferably, HSV-1 and HSV-2 infections, using DNA-based constructs. In one embodiment, the constructs are capable of being packaged *in situ* by an active (replicating) viral infection. In another embodiment, the constructs are amplicons programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). In a preferred embodiment, the constructs are both capable of being packaged *in situ* by a replicating virus and expressing anti-viral compounds.

In a highly preferred embodiment, unpackaged amplicon DNA is applied to the site of an active HSV infection and packaged *in situ*. Consequently, amplicon DNA is incorporated into nascent virus particles in lieu of viral genomes. By reducing the concentration of capsids available to the replicating virus, *in situ* packaging of the amplicon attenuates the infection, decreases infectivity and hastens lesion healing. Concurrently, amplicon-directed IFN- γ expression interferes with viral replication, attenuates the on-going infection, and accelerates the onset of latency. In a highly preferred embodiment, amplicon DNA also remains resident in neuronal cells, the same as, or near to those which harbor latent HSV. The same environmental signals which reactivate the latent HSV virus also induce the amplicon-directed expression of IFN- γ . Consequently, viral latency is maintained or quickly re-established.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 HSV-1(pIFN- γ) is schematically depicted in Figure 1. HSV-1 sequences include an a' region packaging site; an HSV-1 origin of DNA replication (ori_s); a transcriptional unit (HSV-1 c Region) containing an HSV-1 immediate early gene promoter (IE 4/5 promoter) and intervening sequences following the promoter. Non-HSV features include a pBR322 backbone containing a prokaryotic origin of replication (Col E1 Ori) and the ampicillin resistance conferring sequence (Amp); the SV40 polyadenylation site; and a mouse interferon gamma coding sequence (muIFN- γ).
- Figure 2 Figure 2 compares the total IFN- γ production in the brains of mice infected with the HSV-2(G) strain of HSV-2 (column 1); mice co-infected with HSV-2(G) and HSV(pIFN- γ) (columns 2-3) DNA; and mice co-infected with HSV-2(G) and control vector DNA, HSV(pLAC) (columns 5-6). Constructs HSV-1(pIFN- γ) and HSV-1(pLac) are designated HSV(pIFN- γ) and HSV(pLac), respectively, in Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994).
- Figure 3 Color photographs of HSV-1 induced herpes labialis in the cotton rat model.
- Figure 4 HSV-1 titers in lips, trigeminal ganglia and brainstem of HSV-1 infected cotton rats.
- Figure 5 Color photograph of immunohistochemically stained samples from cotton rats infected with HSV-1. Dark stain indicates HSV-1 protein detection.
- Figure 6 Schematic representation of commercially available pBK-CMV phagemid.
- Figure 7 Schematic representation of amplicon HSV-2(pCMV).
- Figure 8 Schematic representation of amplicon HSV-2(pCMV-LacZ).
- Figure 9 Schematic representation of amplicon HSV-2(pCMV-muIFN- γ).
- Figure 10 Schematic representation of amplicon HSV-2(pfos-muIFN- γ).
- Figure 11 Schematic representation of amplicon HSV-2(pCMV-crIFN- γ).
- Figure 12 Color photographs illustrating the reduction in oral lesion formation upon topical application of HSV-2(pCMV) in the cotton rat model.

DETAILED DESCRIPTION OF THE INVENTION

While not intending to be bound to any one theory in the practice of this invention, the expression vectors, treatment systems, and methods described herein are believed to rely on well known properties of viral replication known to be shared among many viral families of large DNA viruses. Although one of skill in the art will recognize that the present invention may be used to treat a host of infectious viral diseases, in the interests of brevity, the Examples 1-13 are confined to Herpesvirus, in particular, HSV-1 and HSV-2. Consequently, this invention may be best understood in light of the current understanding HSV life-cycle. Details of HSV biology may be found in Bernard Roizman and Amy E. Sears, Herpes Simplex Viruses and Their Replication, Fields Virology Ch. 72 (3d. Ed., B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated by reference), and in the other referenced cited herein.

The HSV virion consists of four physical elements: 1) an electron-opaque core, containing approximately 150 kbp of double-stranded, predominantly linear DNA; 2) an icosadeltahedral capsid surrounding the core; 3) an amorphous tegument surrounding the capsid; and 4) a glycoprotein-studded outer lipid envelope.

In clinical infections, the virus attaches to cell-surface receptors, usually at a site of dermal or mucosal abrasion. The viral envelope fuses with the plasma membrane of epithelial cells and the de-enveloped capsid enters the cytoplasm. The capsid is transported to the nuclear pores and the DNA is released into the nucleus where it circularizes. Viral transcription, and capsid assembly take place in the nucleus. New copies of viral DNA are produced by rolling-circle replication, cleaved into monomers and packaged into preformed capsids. Tegument proteins and the lipid envelope are added and mature virus is exported through the endoplasmic reticulum. This active infection process is usually accompanied by the localized cell necrosis, inflammation, and vesicle formation characteristic of clinical lesions. Ultimately, however, the virus is cleared from the inoculation site.

In addition, the HSV viruses are highly neurotropic and can spread from the inoculation site to peripheral sensory nerves. Retrograde transport delivers the viral capsids up the nerve to the dorsal root ganglia. Again, the DNA is released through the

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nuclear pores into the nucleus and circularizes. However, unlike the situation in epithelial cells, the HSV DNA may remain latent, exhibiting little or no detectable transcription or replication. Nevertheless, under certain conditions, this apparently quiescent episomal construct can be reactivated. The DNA replicates and new infectious particles are transported back down the peripheral sensory nerves by anterograde transport. Upon reaching the synapses, infectious particles attach to epithelial cells at or near the initial site of inoculation and re-establish an active mucocutaneous infection.

Both the clearance of mucocutaneous infection and the reactivation of latent virus have long been associated with the immune status of the host. (See e.g., J. Lindsay Whitton and Michael B.A. Oldstone Immune Response to Viruses, Fields Virology Ch. 10, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated herein by reference)). This observation has spawned numerous searches for immunological components involved in virulence. Interferons alpha, beta and gamma have been suggested as treatments for HSV infection. (Hamuy et al., Eur. J. Dermatol. 8:310-9 (1998); Smith et al., Virol. 202:76-88 (1994)).

Recently, it has been discovered that interferons, especially IFN- γ , have a potent inhibitory effect on both HSV replication and transcription termination. (Feduchi, E. et al., Virol. 180:822-825 (1991); Komatsu, T., et al., J. Neuroimmunol. 68:1010-108 (1996); Lewandowski, G., et al., J. Neuroimmunol. 81:58-65 (1998)); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998); Schijns, V., et al., J. Neuroimmunol. 28:1-7 (1990); Svennerholm, B., et al., Arch. Virol. 104:153-156 (1989)). Consequently, the production of one or more interferons at the site of infection would hasten the resolution of an active infection. More importantly, the production of one or more interferons at the site of a latent virus would serve to keep the virus in the latent phase, eliminating or reducing the frequency and/or severity of recurrent episodes and limiting the spread of the virus in the host.

The present invention thus encompasses means for delivering antiviral compounds, preferably cytokines, most preferably IFN- γ , to the site of active and/or latent HSV infection. Although any delivery mechanism is acceptable, it is preferred that

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the antiviral compound is a protein expressed at or near the desired site of action. Acceptable delivery systems include amplicons, viruses, defective viruses or defective interfering viruses (DI particles), or combinations thereof, which have been engineered to express IFN- γ , and/or other efficacious compounds. (See, e.g., Donald M. Coen and Robert F. Ramig, Viral Genetics, Fields Virology Ch. 5, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996); Ho, Methods in Cell Biology 43:191-210 (1994); and U.S. Patent No. 5,672,344, each of which is incorporated herein by reference.)) In a preferred embodiment, the delivery system is an amplicon, more preferably, an amplicon based on HSV-1 or HSV-2 elements.

Specific details of constructing and propagating amplicons based on HSV sequences are available in U.S. Patent Nos. 5,501,979; 5,661,033; 5,672,344; 5,599,691; and in Angela P. Dyer and Frank Tufaro, Herpes Simplex Virus Vectors for Gene Therapy of the Nervous System, Protocols for Neural Cell Culture (2nd ed. S. Fedoroff and A. Richardson Eds., Humana Press, 1997); Ho, Meth. Cell Biol. 43:191-210 (1994); Geller, A. I., et al., Proc. Natl. Acad. Sci. USA 90:7603-7607 (1993); and Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)), each of which is incorporated herein by reference. Additional techniques useful for the construction of amplicons and other DNA constructs may be found in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, (1989), incorporated by reference.

In the present context, an amplicon is a plasmid which contains bacterial plasmid sequences which allow prokaryotic amplification, as well as sequences which allow expression and/or replication in eukaryotic hosts, and signals compatible with a viral packaging system, usually a virus-infected cell monolayer. Amplicons are thus designed to mimic the packaging signals of an intact viral genome such that amplicon DNA replaces the viral DNA in nascent viral capsids. Thus, in the case of HSV, because an HSV virion can package 152-154 kb of DNA (the HSV genome), 31-32 copies of a 4.8 kb HSV-based amplicon vector would be packaged per virion.

Generally, packaging is accomplished *ex vivo* and requires a defective "helper" virus to generate the packaging machinery and capsids. Unfortunately, an inherent

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failing of existing packaging systems is the production of infectious amplicon particles contaminated with helper virus. Consequently, therapies based on standard amplicon/helper virus systems increase the viral load on the host, and may contribute to cytopathic effects. Although techniques such as using conditionally defective viral mutants are designed to reduce cytopathic effects, it is ultimately desirable to avoid exposing the host to any possible additional pathogen.

In a preferred embodiment of the present invention, the amplicons can function without helper viruses and are applied to the site of an active viral infection as unpackaged DNA. Because the amplicon contains signals which mimic those of the actively replicating virus, the exogenous DNA will be packaged into infectious particles *in situ*. Because *in situ* packaging depends on on-going viral replication, the amplicons are only packaged in infected cells. Consequently, the applied DNA is effectively targeted to those specific cells. In addition, because virions containing amplicon vector DNA are indistinguishable from virions containing viral genomes, the packaged amplicon vectors will spread the same as infectious viral particles.

Moreover, with respect to neurotropic viruses such as HSV, the active lesion provides a portal for both the virus and the amplicon to enter the nervous system. Again, because the packaged amplicon is functionally indistinguishable from the packaged virus, it will be transported to the same or similar sites in the peripheral and central nervous system. Consequently, expression of the DNA of the amplicon in neural tissues will occur specifically in HSV-infected regions, thereby increasing the therapeutic effect and greatly decreasing potential side effects.

This embodiment not only avoids the potential dangers to the host, but simplifies production by eliminating the time, effort and expense of helper virus packaging. In addition, this embodiment provides for targeting to the site of active viral infection, and allows targeting to more remote sites of viral residence. Moreover, because this method does not require storage and application of infectious viruses, it provides a more stable and easily formulated medicament.

HSV-based amplicon vectors of the present invention generally contain the following six elements:

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- 1) A backbone vector (for example, pBR322) with a prokaryotic origin of replication, and an antibiotic resistance-conferring gene (usually β -lactamase). This permits amplicon vector replication in bacteria, thereby facilitating amplicon isolation and purification.
- 2) The HSV-1 or HSV-2 a' sequence.
- 3) An HSV-1 or HSV-2 replication origin (either ori_s or ori_L).
- 4) At least one promoter compatible with mammalian cells driving expression of a transgene.
- 5) A transgene.
- 6) A polyadenylation site.

The a' and ori sequences are necessary for the replication and packaging of HSV-based amplicons. The a' sequence serves two functions: HSV replicates by a rolling circle mechanism which produces concatamers of the viral genome; a' provides both the site for cleavage into single genome lengths, and the packaging signal for inserting the unit length DNAs into preformed capsids. The HSV genome consists of two covalently linked components designated L (long) and S (short), each of which contains a corresponding origin of viral replication (ori_s and ori_L , respectively).

Because the a' and ori signals may be poorly recognized by non-type viruses, it is preferred that HSV-1-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-1, whereas HSV-2-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-2. Although in the present examples, HSV ori_s sequences provides an origin of replication, allowing amplicon vector DNA replication in HSV-infected cells, an ori_L sequence may be substituted. Moreover, as recognized by one of ordinary skill, analogous sequences from other viruses may be substituted to affect packaging into actively replicating non-HSV virions and to treat diseases other than HSV-1 and HSV-2.

HSV-based amplicon vectors can be designed with a variety of promoters for constitutive or induced expression of any potential antiviral protein. Preferred promoters fall into two, potentially overlapping, classes: those that will direct expression of the transgene in epithelial cells and those which are active in neuronal cells.

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Given the multiple stages of HSV infection, it is preferred that the anti-viral transgene is constitutively expressed in epithelial cells. Consequently, transgene expression from this promoter will coincide with active infection and diminish as the virus is cleared from the epithelial cells. Transgene expression in the lesion will reduce the severity and duration of an infection, promote healing, and induce the virus to enter latency.

In contrast, to limit adverse neurological consequences, it is preferred that expression in neuronal cells is inducible. Ideally, expression in neuronal cells is stimulated by the same signals which stimulate neuronal HSV to break latency and begin replicating. The amplicon will tend to remain in neurons in a 'latent' state similar to HSV, nevertheless, the amplicon-driven transgene will be expressed at the same time that HSV is replicating in the nervous system. Consequently, expression of antiviral compounds in neuronal cells will tend to preserve latency and limit the frequency and/or severity of recurrent herpetic outbreaks.

Thus, the preferred embodiments of the invention comprise DNA-based therapies for HSV infections that will be effective during symptomatic and asymptomatic episodes. Most notably, the inducible neuronal expression of anti-viral proteins will maintain HSV in a virtual latent state. Although expression may be regulated, for example, by using a tetracycline-responsive promoter (Fotaki et al., Gene Therapy 4:901-08 (1997)), it is preferred that the neuronal expression is stress inducible. Any stress-inducible host or viral promoter may be used including, but not limited to, promoters for glucose regulated proteins (grps), inducible heat shock proteins such as *hsc70*, *hsp70* and *hsp90*, *c-jun*, *c-fos*. In a preferred embodiment, the *c-fos* gene promoter, which is activated during stress and primary HSV-1 infections, is used to drive inducible neuronal expression of anti-viral proteins. (Gieroba et al., Brain Res. 675:329-32 (1995); Senba et al., Neurosci. Res. 29:183-207 (1997); Sharp et al., Neurotoxicol. 15:51-59 (1994); and Tyrell, EXS. 77:255-71 (1996)).

The amplicons of the invention are programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). Other useful anti-viral compounds which may be expressed alone or in combination with

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themselves, and/or with IFN- γ , include IFN- α , IFN- β , TNF- α , TNF- β , TGF- α , and anti-viral antibodies. (Wong et al., Nature 323:819-822 (1986); Lu et al., J. Invest. Dermatol. 108:803-808 (1997); Kumano et al., Antiviral Res. 7:289-301 (1987); and Yamada et al., Arch. Virol. 99:101-109 (1988)).

One highly preferred embodiment comprises a HSV-1 or HSV-2-based amplicon containing: 1) a constitutive CMV immediate early gene promoter driving IFN- γ expression; and 2) a second HSV-based amplicon of the same HSV type, containing the stress-inducible c-fos gene promoter driving IFN- γ expression. Alternatively, both the constitutive and stress- promoters may be constructed on a single HSV-1 or HSV-2-based amplicon.

In this highly preferred embodiment, inoculation at the site of viral replication initiates a four tier anti-viral therapy:

First, IFN- γ expression from the CMV promoter exerts an antiviral effect at the site of active infection. Increasing IFN- γ levels repress the CMV promoter reducing amplicon-directed IFN- γ production in the epithelial cells. However, because IFN- γ protein has a half-life of 48 hours, it continues to exert an antiviral effect for the duration of the outbreak.

Second, the replication machinery of the resident virus packages the amplicon in HSV virions *in situ*. Each virion that packages amplicon DNA becomes replication-deficient and reduces the viral load on the host. Moreover, the infectious titer at the mucosal membrane is decreased, thereby reducing the likelihood of transmission.

Third, HSV virions containing amplicon DNA are transported into the nervous system. The expression of IFN- γ in the nervous system expedites HSV replication termination and establishment of latency.

Fourth, as the host is exposed to emotional or physical stress (sunlight, fever, immunosuppression, etc.), cellular events occur that cause the latent HSV to reactivate and begin to replicate. The same stressors that reactivate latent HSV will activate the c-fos gene promoter in the "latent" HSV-based amplicon vector. The activated c-fos promoter will drive IFN- γ production, which will rapidly terminate the HSV DNA replication cycle. Consequently, no HSV particles will be made, and HSV will quickly

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return to a latent state. This will potentially keep HSV in a virtual latent state and block asymptomatic shedding of the virus.

The amplicon DNAs of the present invention may be applied to any stage of an active, primary or recurrent, episode of herpes labialis or genitalis, including vesicular (papule, vesicle, and pustule) and crusting stages. However, it is preferred that the DNA be applied early in the virulence cycle during prodrome (throbbing-tingling) and/or erythema (macule) stages. Although topical application is preferred, the amplicon may be administered by any method including subcutaneously, intradermally, intravenously, intraperitoneally, by inhalation or lavage, orally, mucosally, intranasally, or by targeted injection into a lesion or affected body site, including dermal and mucosal tissues, eye, brain, ganglia and other neural tissues.

The DNA-containing compositions of the invention may be applied alone, complexed, suspended, or dissolved in a carrier. An efficacious amount of a DNA-containing composition and carrier comprises a preparation for the treatment of a viral disease or condition. Acceptable liquid carriers or carrier components include: water, polyethylene glycol, dimethyl sulfoxide (DMSO), oils, (including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil), or mixtures thereof, alone or in combination. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., Mack Pub., Easton, Pa., 1990), incorporated by reference. Carriers can be in the form of mists, sprays, powders, waxes, cremes, salves, ointments, patches, poultices, films, or cosmetic preparations. The DNA composition may also include topical anesthetics such as lidocaine, xylocaine and the like, flavorants, emulsifiers, antibiotics, and known or suspected anti-viral compounds including, but not limited to, idoxuridine, interferons, caffeine, anti-viral antibodies, glutaraldehyde, trifluorothymidine (TFT), nonoxynol-9, acyclovir, penciclovir, famciclovir, valaciclovir, cidofovir, brivudin, sorivudine, n-Docosanol, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC), arildone, penciclovir, ribonucleotide reductase inhibitors, SP-303 from *Croton iechler*, Foscarnet, tromantadine, or 2-(N,N-dialylaminomethylene)-9[[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl]guanine (U.S.

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Patent No. 4,461,757), tumour necrosis factors alpha and/or beta, ascorbic acid, povidone-iodine, and the like.

Because the amplicon DNAs of the invention are preferably administered topically, it is preferred that the DNA is complexed with, dissolved, or suspended in a delivery vehicle that both facilitates DNA transfer through the stratum corneum to the living cells of the lower epidermis, and promotes the uptake of the DNA into the cells. Since 1966, DMSO, an absorption-enhancing agent, has been investigated as a topical delivery system for nucleoside analogs (Becker, Y., *Virus Genes* 10:217-226 (1995)). In Europe, idoxuridine (IDU) in DMSO (80%) is currently in use for the treatment of herpes labialis, genitalis, and zoster. PEI is a cationic polymer that acts as a DNA "sponge" and efficiently delivers DNA into cells. PEI and similar polycationic polymers can be used to transfect DNA into cells both in vitro and in vivo (Abdallah, B., A., et al., *Human Gene Ther.* 7:1947-1954 (1990); Boletta, A., et al., *Human Gene Ther.* 8:1243-1251 (1997); Boussif, O., et al., *Proc. Natl. Acad. Sci. USA* 92:7297-7301 (1997); Goldman et al., *Nature Biotech.* 15:462-467 (1997)), and has been used to deliver HSV-based amplicon DNA into HSV-infected ocular cells in mice. (Ho, D. Y., *Methods in Cell Biol.* 43:191-210 (1994); Lewandowski, G., et al., *J. Neuroimmunol.* 81:66-75 (1994)). Consequently, preferred carriers comprise DMSO and PEI, alone or in combination.

The invention also relates to the treatment of a patient or organism by administration of an efficacious amount of the DNA compositions described herein. A patient or organism is hereby defined as any person or non-human subject in an experimental model, any person or non-human animal in need of anti-viral therapy, or any subject for whom treatment may be beneficial, including humans and non-human animals. Such non-human animals to be treated include all domesticated and feral vertebrates, preferably but are not limited to mice, rats, fish, including carp, pike, catfish, walleye, turbot and salmon, birds, including ducks, pigeons, chickens, and turkeys, rabbits, hamsters, goats, guinea pigs, deer, donkeys, dogs, cats, swine, horses, cattle, sheep, turtles, lizards, snakes, and non-human primates, including monkeys, macaques, marmosets, chimpanzees, orangutans, gorillas and marmosets.

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Treatment comprises effecting any observable or measurable anti-viral effect in a patient or organism. Observable or measurable anti-viral effects include, but are not limited to, reducing viral titer; decreasing viral shedding and infectivity; preventing, limiting, or retarding the spread of the virus in an infected organism; eliminating or reducing the severity and/or duration of an active viral infection, accelerating healing of virus-induced lesions or conditions, promoting, inducing, maintaining or reinforcing viral latency; reducing mortality rates associated with viral infection; and providing prophylactic protection from viral disease, or the effects of a viral disease, by administering the DNA compositions of the invention to an uninfected patient or organism.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

Example 1

***In situ* packaging of HSV-based amplicon DNA into HSV virions**

To demonstrate that unpackaged DNA can be packaged *in situ* by an active viral infection, we inoculated DNA of the HSV-1-based amplicon vector HSV-1(pIFN- γ) (Figure 1) into the eyes of HSV-2 infected mice. The construction of HSV-1(pIFN- γ) and the intraocular model of infection are described in detail in Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998) and Lewandowski, G., et al., J. Neuroimmunol. 55:23-34 (1994), respectively. Briefly, the vitreous compartments of 25 BALB.cByJ mice (6-8 weeks of age) were infected with HSV-2(G) (2×10^4 pfu = $2300 \times \text{LD}_{50}$). HSV-1(pIFN- γ) DNA was administered intraocularly to ten mice at 6h pre-inoculation and to five mice at 16h postinoculation. The inoculi were formulated to contain 3 μg of an HSV-1-based amplicon plasmid expressing IFN- γ (HSV-1(pIFN- γ) mixed with (i) 5% glucose-saline, or (ii) 9 equivalents of polyethylenimine (PEI), a transfection agent, in the same vehicle.

As expected, the ten HSV-2-infected mice treated with vehicle only died by day 5. By contrast, the five HSV-2-infected mice treated postinoculation with either HSV-1(pIFN- γ) DNA formulation survived 9-10 days. Of the ten HSV-2-infected mice treated with HSV-1(pIFN- γ) DNA pre-inoculation, all survived at least 11 days and 33%

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completely recovered. These results indicated that a single administration of HSV-based amplicon plasmid (DNA) expressing IFN- γ could prolong survival of HSV-2-infected mice.

Although the increased survival of HSV-1(pIFN- γ)-treated animals suggests that the amplicon may have been packaged *in situ* by the actively replicating HSV-2 virus, *in vitro* studies (not shown) indicate that HSV-2 virions inefficiently package and replicate amplicon based on HSV-1 DNA. This is most likely due to the mismatch of HSV-2 virions with HSV-1 α' and/or HSV-1 ori_s sequences.

Example 2

HSV-based amplicon DNA is transported into the nervous system where it expresses high levels of IFN- γ

HSV-1(pIFN- γ)-containing virions have previously been demonstrated to be transported from the eye to the brain of HSV-2-infected mice (Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). To demonstrate neuronal transport and expression, HSV-1(pIFN- γ) and HSV-1(pLac) DNAs (Geller and Breakefield, Science 241:1667-79 (1988), were packaged into HSV-1 virions *in vitro* (Ho, D. Y., Methods in Cell Biol. 43:191-210 (1994); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). Mice were then co-inoculated with amplicon DNA and HSV-2 virus by direct injection into the vitreous compartment of the eye. At various times postinfection IFN- γ production was measured in the brain using an ELISA kit (Genzyme Corp., Kendall, MA). An acceptable ELISA assay is also described in Antibodies: A Laboratory Manual, (Harlow & Lane eds., 1988), Cold Spring Harbor Laboratory Press (incorporated by reference).

As shown in Figure 2, very little IFN- γ was detected in the brain during HSV-2-infection (hatched bar). However, high IFN- γ levels were detected in the brains of HSV-2-infected mice co-inoculated with HSV-1(pIFN- γ) (solid bars), strongly suggesting that HSV virions containing HSV-based amplicon vector DNA are transported from the periphery into the nervous system.

Example 3**Experimental models of herpes labialis and herpes genitalis.**

Numerous models of HSV pathogenesis are known in the art and are briefly described in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d. ed., Ch. 73, each of which may be used in the practice and refinement of this invention. Although the ocular model used in the above demonstrations is useful for studying certain neurotropic effects, it provides only a limited representation of the clinical course of HSV infections. Moreover, rapid destruction of non-replicating retinal cells in the HSV-2 ocular model severely limits HSV-based amplicon DNA cellular uptake and forbids multiple applications of HSV-based amplicon vector DNA. Consequently, this application presents novel experimental models of herpes labialis and herpes genitalis in cotton rats and mice which are particularly useful in describing the invention disclosed herein.

Herpes labialis models were established in inbred male and female Sigmodon hispidus cotton rats and BALB.cByJ mice (6-8 weeks of age). Although both mice and cotton rats are susceptible to herpes labialis, infectious virus titers in the mouse nervous system are higher than those in the rat nervous system. In addition, the process of HSV-1 induced lesion formation and healing in mice requires >15 days, as compared to 10 days in the rat. Consequently, the progression of herpes labialis (shedding, lesion formation, and resolution) in cotton rats provides a better model for clinical human disease.

Although the time course of the herpes labialis in cotton rats closely simulates human condition, the wealth of immunological and neurobiological reagents for mice enable a more detailed examination of changes in the immune response and nervous system after application of the instant constructs. Consequently, information obtained by testing the constructs in both systems may be useful for the design of safe and efficacious human treatments. Such treatments may be analyzed in both clinical studies and in human modes of labial and genital herpes such as those described in Rooney et al., Lancet 338:1419-22 (1991) and Rooney et. al., J. Infect Dis. 166:500-506 (1992), both of which are incorporated by reference.

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Animals are anesthetized by methoxyflurane inhalation and the center of the lower lip is scratched with a 21-gauge needle. The scratched area is swiped with a sterile cotton swab soaked in HSV-1 inoculum (1×10^7 pfu/ml). The animals are then characterized with respect to: (1) lesion formation and healing, (2) infectious virus recovery from lips, trigeminal ganglia and brains, and (3) immunohistochemical detection of HSV antigens in lips, trigeminal ganglia and brains

Example 4

HSV-1 induced herpes labialis in cotton rats and mice

The progression of HSV-1 induced herpes labialis in the cotton rat model is shown in Figure 3. The prodrome and erythema stages occurred at 1d-2d (days postinoculation) (Panel A). Lip lesions were first visible at 2d-3d (Panel B), and progressed to the vesicle stage between 4d and 5d (Panel C). Finally, the lesions began crusting at 5d-6d (Panel D), and healing was nearly complete by 9d-10d (Panels D and E).

HSV-1 titers in lips, trigeminal ganglia and brainstem were determined by standard plaque assay and the results plotted in Figure 4. HSV titers in the lips peaked at 2d (Panel A). The sharp decrease in HSV-1 titers at 3d was indicative of the viral replication termination. The low HSV titers at 5d-7d coincided with viral clearance. These data are in good agreement with the clinical stages of herpes labialis. Specifically, HSV replication and shedding were rapid, and occurred during the first 2-3 days, whereas viral clearance and lesion healing required an additional 7 days.

HSV replication in the trigeminal ganglia began at 2d, peaked at 4d, and declined sharply at 6d (Panel B). In the brainstem, HSV replication occurred through 5d, and was decreased by 6d (Panel C). Infectious HSV was no longer detectable in the trigeminal ganglia and brain at 14d. The HSV recovery time courses suggest that HSV travels from the lip into the trigeminal ganglia, and then from the ganglia into the brain.

Immunohistochemical analysis of excised tissues demonstrates the expression of HSV-1 proteins in infected tissue (Figure 5). HSV proteins are abundant in the lip at 1d (Panel A), and are still detectable at 5d during lesion healing (Panel B). As

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anticipated, at 5d, we detected HSV in the trigeminal ganglia neurons (Panel C), and tract (Panel D). Unexpectedly, we also detected HSV in the brainstem (Panel E) and cerebellum (Panel F).

By 30d HSV establishes a latent infection in the trigeminal ganglia, and brain. The latent infection can be reactivated in cotton rats by immunosuppression with cyclophosphamide (50 mg/kg) and dexamethasone (8 mg/kg). In the cotton rat model, HSV titers were first detected 8 days post- treatment in lips, trigeminal ganglia, and brainstem. (Data not shown).

Similar results were obtained using BALB.cByJ mice. From these data we conclude that HSV inoculation of cotton rats and mice provide excellent models of herpes labialis primary and recurrent infections.

Example 5

Experimental models of HSV-2 induced herpes genitalis

Sigmodon hispidus cotton rats and BALB.cByJ mice are also susceptible to intravaginal HSV-2 inoculation. Female animals (6-10 weeks) are anesthetized by intraperitoneal injections of acepromazine (2.5 mg/kg) and ketamine (25 mg/kg), and intravaginally inoculated with HSV-2(G) (1×10^7 pfu/ml) (20 μ l mice, 75 μ l rats). Adult female mice are susceptible to vaginal HSV-2 during diestrus and pregnancy, but not at estrus. However, with progestin treatment, the animals are continually susceptible to HSV-2. (Baker, D. A. and S. A. Plotkin. 1978). Vaginal infection in mice by herpes simplex type II is enhanced with progesterone. (Proc. Soc. Exp. Biol. Med. 158:131-134; Baker, D. A., et al., et al., Proc. Soc. Exp. Biol. Med. 158:131-134; Parr, M. B., et al.; Lab. Invest. 70:369-380 (1994)). Thus, female cotton rats and mice are injected subcutaneously with 0.01 μ g of estradiol benzoate in peanut oil, followed 24 h later by subcutaneous injection of 2.0 mg of progestin (Depo-Provera, Upjohn, Kalamazoo, MI) in PBS. The animals are inoculated 5 days later.

In the absence of progestin treatment, 40%-60% of HSV-2-inoculated animals became infected. Progestin treatment increased the susceptibility to intravaginal HSV-2 to 100%. We observed 100% morbidity and mortality in the HSV-2-infected animals.

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HSV was recovered from vaginal washes beginning at 1 dpi, and from dorsal root ganglia and spinal cord beginning at 3 dpi. Although the high mortality rate makes this model difficult, it is an excellent paradigm for efficacy testing of anti-herpes therapies.

Example 6

Construction of HSV-2-based amplicon vectors

HSV-2(pCMV)

The HSV-2(pCMV) amplicon vector was constructed as follows: The TK poly(A), neomycin/kanomycin resistance gene (Neo R), and SV40 origin of replication (SV40 ori) sequences of the pBK-CMV phagemid (Stratagene, LaJolla, CA) (Figure 6), were replaced with the β -lactamase promoter and gene (Amp^R) to generate plasmid pBK-CMV/Amp^R. The HSV-2(G) packaging signal sequence (a' sequence, 550 bp), and origin of replication (ori_s, 667 bp) were amplified from HSV-2(G) genomic DNA (ABI, Gaithersburg, MD) using the polymerase chain reaction (PCR). Each sequence was subcloned into the pCR-Script vector. The HSV-2(G) a' sequence was then cloned into the MluI site of pBK-CMV/Amp^R by blunt-end ligation. Likewise, HSV-2(G) ori_s DNA was cloned into the BspLu11I site, to generate amplicon HSV-2(pCMV) (Figure 7).

HSV-2(pCMV-LacZ)

To remove the bacterial LacZ promoter and a portion of the polylinker, HSV-2(pCMV) will be incubated with endonucleases Sal I and Nhe I. Then the psv- β -galactosidase vector (Promega); which contains the full-length β -gal gene, will be digested with the Hind III endonuclease. The Nhe I and Sal psv- β -gal vector ends will be modified to have blunt ends, and ligated to Nhe I linkers (Stratagene). The modified psv-ga β vector will be digested with Nhe I and Sal I and ligated with the NheI/Sal I-digested HSV-2(pCMV) vector to create the reporter construct HSV-2(pCMV-LacZ) (Figure 8).

HSV-2(pfos-LacZ)

HSV-2(pCMV-LacZ) will be digested with endonucleases Nsi I and Nhe I. The mouse c-fos promoter will be amplified from mouse (BALB.c) brain DNA using PCR. The forward and reverse primers (Life Technologies, Gaithersburg, MD) are engineered such that the amplified c-fos promoter sequence will contain a 5' NsiI site and a 3' NheI

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site. The amplified c-fos promoter will be ligated with the NsiI/NheI digested HSV-2(pCMV-LacZ) amplicon vector, to generate the stress inducible reporter construct HSV-2(pfos-LacZ).

HSV-2(pCMV-mulFN- γ)

The β -gal gene will be removed from the HSV-2(pCMV-LacZ) vector by blunt end digestion with endonucleases Nhe I and Pvu II. The mouse IFN- γ coding sequence will be amplified from the vector pms10 (ATCC, Manassas, VA), using PCR. However, and as understood by those of skill in the art, human IFN- γ sequences could be amplified to provide human therapeutic amplicons. The forward and reverse primers are engineered such that the amplified IFN- γ gene coding sequence will have a 5' Nhe I site and a 3' Dra I blunt end. The amplified mouse IFN- γ will be ligated with the Nhe I-Pvu II digested vector to generate HSV-2(pCMV-mulFN- γ) (Figure 9).

HSV-2(pfos-mulFN γ)

The CMV promoter in HSV-2(pCMV-mulFN- γ) will be replaced with the mouse c-fos promoter, described above, to generate HSV-2(pfos-mulFN γ) (Figure 10).

HSV-2(pCMV-crIFN γ)

The cotton rat (*Sigmodon hispidus*) IFN- γ coding sequence (crIFN- γ) will be amplified using PCR from a cotton rat cDNA library. The forward and reverse primers will be engineered such that the amplified crIFN- γ gene will have a 5' Nhe I site and a Dra I blunt 3' end. As described in above, the β -gal gene will be removed from HSV-2(pCMV-LacZ), but replaced with the crIFN γ gene, to generate HSV-2(pCMV-crIFN γ) (Figure 11).

HSV-2(pfos-crIFN- γ)

The CMV promoter will be removed from the HSV-2(pCMV-crIFN γ) vector, and replaced with the mouse c-fos promoter, described above, to generate the HSV-2(pfos-crIFN- γ) vector.

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Example 7**Construction of HSV-1-based amplicon vectors**

HSV-1-based amplicon vectors are constructed essentially as described for the HSV-2-based amplicon vectors from pBK-CMV/Amp^R except that the HSV-1 a' sequence and ori_s sequences are amplified from HSV-1(F) virion DNA (ABI) and used in lieu of HSV-2 sequences.

Example 8**In vitro testing of amplicon constructs**

The HSV-1 and HSV-2-based amplicon constructs may be transfected into Vero cells and tested for the production of expression products using standard methods. For example, IFN- γ production from HSV-2(pCMV-mulFN γ) and HSV-2(pfos-mulFN γ) vector DNA's are measured at 24h using an ELISA kit from Genzyme. Similarly, IFN- γ production from HSV-2(pCMV-crIFN γ) and HSV-2(pfos-crIFN γ) vector DNA's is measured at 24h with an ELISA assay optimized to detect *Sigmodon hispidus* IFN- γ .

Example 9**HSV-2(pCMV) is packaged into HSV-2 virions in vitro**

Vero cells were transfected with HSV-2(pCMV) DNA from 10 clones, and then 24h later infected the cells with HSV-2(G) (0.01 pfu/cell). At 48h postinfection, the culture supernatants were transferred to fresh Vero monolayers. After 24h, total DNA was isolated from the cells and samples were then screened for the β -lactamase gene by PCR. The presence of the β -lactamase gene in all clones indicated that the HSV-2-based amplicon DNA was successfully packaged and replicated in HSV-2(G) virions.

Example 10**Optimizing the administration of HSV-based amplicon vector DNA, and *in situ* packaging into HSV virions.**

The HSV-based amplicon constructs described above contain both the DNA packaging/cleavage signal and the HSV ori_s. Thus, the HSV virions in herpetic lesions will package topically applied HSV-based amplicon DNA. Each HSV virion can package

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152-154 kb of DNA; thus, several copies of an HSV-based amplicon vector could be packaged per virion. Clearly, the level of transgene expression (e.g., β -gal or IFN- γ) will depend on the efficiency of HSV-based amplicon DNA packaging into HSV virions. Moreover, the therapeutic effect of the HSV-based amplicon will depend on the level of transgene expression. Consequently, it is desirable to optimize the conditions for *in situ* packaging in the cotton rat and mouse model systems. This will entail determining: 1) an optimal vehicle for topical DNA delivery; 2) the optimal timing and dosing schedule (e.g., prodrome, erythema, papule, vesicle, pustule, crusting, or scab, single vs multiple applications); and 3) the amount in μ g of HSV-based amplicon vector DNA that is most efficiently packaged.

The results obtained using the model systems can then be extrapolated to human patients and used to plan safety and efficacy studies for the topical treatment of herpes labialis and genitalis and related conditions.

Vehicles for DNA delivery

The following experiments provide guidelines for testing the efficacy of DMSO and PEI carriers using the HSV-1-based system. The same basic experiment will be done with the HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, (without virus) and may be modified to test the efficacy of any other formulation.

Topical administration of HSV-based amplicon DNA in DMSO

To determine if DMSO will enhance the absorption and penetration of topical HSV-based amplicon DNA, HSV-1(pCMV-LacZ) and HSV-2(pCMV-LacZ) DNA in various carriers will be applied to uninfected animals with intact lips. Each application will contain a high concentration (5 μ g) of amplicon DNA in order to increase the signal for low degrees of penetration.

Animals will be anesthetized by intraperitoneal injections of ketamine (25 mg/kg) and acepromazine maleate (2.5 mg/kg). 5 μ l of the following formulations will then be applied to a small area in the center of the lower lips of cotton rats and mice.

Group 1: DMSO (80%)/saline (20%);

Groups 2 & 6: HSV-1(pCMV-LacZ) DNA (2), and HSV-2(pCMV-LacZ) DNA (6) in saline;

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Groups 3-5: HSV-1(pCMV-LacZ) DNA in (3) DMSO (80%)/saline (20%); (4) DMSO (40%)/saline (60%); (5) DMSO (10%)/saline (90%);

Groups 7-9: HSV-2(pCMV-LacZ) DNA in (7) DMSO (80%)/saline (20%); (8) DMSO (40%)/saline (60%), and (9) DMSO (10%)/saline (90%).

10 animals in each group will be analyzed at 0.5, 2, 6, 24, and 48 hours post-treatment. Animals will be sacrificed by CO₂ inhalation, and the lower lips removed. The excised lip tissues will be bisected and fixed in a 0.005% glutaraldehyde/2% paraformaldehyde solution for 1 hour at 4°C. After washing in PBS, the tissues will be placed in a β -gal staining solution (35 mM ferricyanide, 35 mM ferrocyanide, 2 mM MgCl₂, 0.02% Tween, 0.02% Nonidet P-40, 1mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactosidase) overnight at 37°C. After the tissues are washed with PBS, blue staining in the tissue will indicate the β -gal expression from the HSV(pCMV-LacZ) constructs. The lateral and ventral distances between the farthest blue staining and the application site will be measured. These values will be used to determine the diffusion/penetration rates and distances corresponding to each formulation. The ANOVA statistical test will be used to determine whether any significant differences occur between formulations. 10 μ m cryostat sections from the tissues will be mounted on charged microscope slides and incubated for 30 min. in the β -gal staining solution. β -gal expression and histopathological changes in the tissues will be monitored by light microscopy. An optimal delivery system will be the formulation that significantly increases the penetration distance from the application site.

Topical administration of HSV-based amplicon DNA complexed with PEI

An increase in cellular uptake will increase the amount of amplicon DNA available to be packaged by HSV virions. The amplicon DNA (5 μ g) will be mixed with various PEI equivalents (PEI equivalents are based on the nitrogen content of the polymer) ten minutes before application to allow DNA-PEI complex formation. A small scratch centered on the lower lips of anesthetized animals will be made with a 21-gauge needle. The following DNA-PEI formulations will be applied to the scratched areas.

Groups 1 & 6: HSV-1(pCMV-LacZ) (1), and HSV-2(pCMV-LacZ) (6) in saline;

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Groups 2-5: HSV-1(pCMV-LacZ) with: (2) 5 PEI equivalents (equ.), (3) 10 PEI equ., (4) 15 P, EI equ., (5): 20 PEI equ.;

Groups 7-10: HSV-2(pCMV-LacZ) with: (7) 5 PEI equ., (8) 10 PEI equ., (9) 15 PEI equ., (10) 20 PEI equ.

Ten animals will be evaluated per group at 24, 48, and 72 hours post-application. Animals will be sacrificed by CO₂ inhalation, and the lower lips will be removed. β -gal expression will be used as an indication of HSV-based amplicon DNA uptake and expression. Five dissected lips per group will be processed for β -gal expression, and prepared for microscopic analysis as described above. Using a light microscope equipped with a 35-mm camera we will obtain photographs and count the blue-stained cells in each sample. β -gal activity will be quantified in the other 5 dissected lips per group. A 10% homogenate (weight/vol.) will be prepared from the dissected lips in phosphate buffer (pH 7.4). After 2 freeze-thaw cycles, cellular debris will be removed by centrifugation. β -gal activity in the resulting sample supernatants will be measured using a colorimetric assay kit (Promega). Sample proteins will be measured using the BioRad assay and β -gal activities calculated as units per mg protein. Data from cell counts and β -gal activity assays will be analyzed using the ANOVA statistical test. Data from groups 1 and 6 will establish the baseline values for cellular uptake of amplicon DNA without vehicle. Cellular uptake values obtain from groups 2-5, and 7-9 will be compared to the baseline values, and will indicate whether PEI offers any improvement over the use saline alone.

Topical administration of HSV-based amplicon DNA complexed with PEI

Once the optimal (Opt.) concentrations of DMSO and PEI carriers have been determined from the above experiments, uninfected cotton rats and mice with intact lips will be tested to determine whether DMSO can facilitate the penetration and cellular uptake of DNA-PEI complexes, without artificially disrupting the lip surface. 5 ug of DNA will be applied to the lower lip of anesthetized animals with a soft applicator brush. The following formulations will be tested.

Group 1: DMSO (Opt.)/PEI (Opt.)/saline;

Groups 2-5: HSV-1(pCMV-LacZ) in (2) DMSO (Opt.)/PEI (Opt.)/saline; (3) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (4) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (5)

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DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 6-9: HSV-2(pCMV-LacZ) in (6) DMSO (Opt.)/PEI (Opt.)/saline; (7) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (8) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (9) DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 10-11: HSV-1(pCMV-LacZ) in (10) DMSO (Opt.)/ saline; (11) PEI (Opt.)/saline; Groups 12-13: HSV-2(pCMV-LacZ) in (12) DMSO (Opt.)/ saline; (13) PEI (Opt.)/saline. (0.5 Opt. Refers to one-half of the optimal concentration)

Ten animals will be evaluated per group at 2, 6, 24, 48, and 72 hours post-application. Animals will be sacrificed by CO₂ inhalation, and the lower lips will be removed. Data will be collected on: (a) diffusion/penetration distances, (b) qualitative and quantitative analysis of cellular β -gal expression, and (c) β -gal activity levels in tissue homogenates.

Five animals per group will be used to obtain the diffusion/penetration distances and cellular uptake data, and 5 animals will be used for β -gal activity assays. Once again, the penetration distances will indicate the ability of the vehicle to deliver the HSV-based amplicon DNA through the stratum corneum to the living cells. β -gal expression will indicate the ability of the vehicle to enhance cellular uptake of the HSV-based amplicon DNA. Histological data from group 1 will establish whether the vehicle causes severe skin irritation or histopathology. Data from groups 10-13 will establish the baseline values for DMSO and PEI formulations alone. Data obtained from groups 2-9 will be compared to the baseline values and will indicate whether the combination of DMSO and PEI offers any improvement over the use of either vehicle alone. The ANOVA statistical test will be used to determine if any significant differences occur between the formulations.

The basic experiments described above are for the HSV-1(pCMV-LacZ), and HSV-2(pCMV-LacZ) constructs in the labialis cotton rat and mouse models, without HSV. The results of these experiments will be uncomplicated by the presence of herpetic lesion. However, the presence of a herpetic prodrome or lesion may change the requirements of the vehicle formulation. Also, the topical delivery vehicle formulation may differ somewhat for herpes genitalis. Accordingly, the same basic experiments will be done with the following variations: (1) HSV-1(pCMV-LacZ) construct with HSV-1(F)

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in the labialis models, (2) HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, without virus, and (3) HSV-2(pCMV-LacZ) construct with HSV-2(G) in the genitalis models.

Optimization of dosing schedule of HSV-based DNA amplicon for *in situ* packaging.

Herpes labialis and genitalis lesions occur in 8 stages. Infectious virus is present from the prodromal stage through the pustule (late vesicular) stage. However, current topical treatments of HSV-1 and HSV-2 infections administered after early prodromal or macule (erythema) stages are relatively ineffective (Hamuy, R., et al., Eur. J. Dermatol. 8:310-319 (1998); Spruance, S. L., In E. DeClercq (ed.), Martinus Nijhoff Publishing, p. 69-86 (1998); Spruance, S. et al., J. Infect. Dis. 161:191-197 (1990)). Because the HSV-based amplicon DNA therapy of the present invention preferably incorporates *in situ* HSV-based amplicon DNA packaging by HSV virions, the instant therapy may be effective from the prodromal stage through the vesicular stage.

To determine an optimal dosing schedule, 5 µg of HSV-1(pCMV-LacZ) DNA will be prepared in an optimal delivery vehicle and administered at various times pre- and post-HSV-1(F) inoculation. The HSV-1(pCMV-LacZ) in vehicle will be applied to a small area in the lower lip center of anesthetized animals at the following times.

Groups 1-3: (1) 16h pre-inoculation, (2) 6h pre-inoculation, and (3) 2h pre-inoculation. Pre-inoculation applications will allow cellular uptake of the HSV-1(pCMV-LacZ) DNA before the virus is introduced. This is similar to the HSV-based amplicon DNA packaging protocols used in tissue culture.

Groups 4-5: (4) 2h post-inoculation, and (5) 12h post-inoculation. These groups will represent the prodrome stage of herpes labialis; HSV is present and beginning to replicate, but a lesion is not observed.

Groups 6-10: (6) 24h post-inoculation, (7) 48h post-inoculation, (8) 72h post-inoculation, (9) 96h post-inoculation, and (10) 120h post-inoculation. These groups will represent the progressive stages of herpes labialis.

Group 11: 2h pro-mock inoculation. This group will serve as the negative control for *in situ* packaging by HSV virions.

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Groups 1-3, & 11 will be sacrificed 48h after inoculation. Groups 4-10 will be sacrificed 48h after amplicon DNA application. The lips will be removed and tissue homogenates (10% wt./vol.) will be prepared in RPMI 1640 with 2% FBS, as described above. All of the sample supernatants will contain HSV-1 virions with infectious DNA. The supernatants will also contain HSV-1 virions with amplicon DNA, if *in situ* packaging of the HSV-1(pCMV-LacZ) DNA has occurred. We will plate the sample supernatants onto confluent Vero cell monolayers in 96 well plates in triplicate. The plates will be placed at 37°C for 48-72 hours, and then processed for β -gal activities and protein concentrations. Because of the large number of samples to process, data collection will be facilitated by use of a microtiter plate reader set at a wavelength of 420 nm. β -gal activities for each group will be compared and analyzed using the ANOVA statistical test.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. As recognized one of skill in the art, the same basic tests may be done with any number of variations including: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Optimizing the amount of HSV-based amplicon DNA amount for *in situ* packaging

Ideally, the absolute amount HSV-based amplicon DNA that can be packaged *in situ* depends on the DNA to HSV virion ratio. In accord with the results of the preceding experiments, the following amounts of HSV-1(pCMV-LacZ) DNA prepared in the optimal delivery vehicle will be applied to cotton rats and mice.

Group 1: 0.0 μ g;

Groups 2-7: (2) 0.5 μ g; (3) 1.0 μ g; (4) 5.0 μ g; (5) 10 μ g; (6) 50 μ g; (7) 100 μ g;

Groups 8-13: The same as groups 2-7, except that HSV-1(pCMV-LacZ) DNA will be applied at a different time post-HSV inoculation.

Ten animals per group will be sacrificed at a predetermined time point post HSV-1(pCMV-LacZ) DNA application. The lips will be removed and tissue homogenates prepared. The supernatants will be analyzed for the presence of HSV virions containing HSV1(pCMV-LacZ) DNA on Vero indicator cells. The β -gal activity values will be plotted

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against the HSV-based amplicon DNA doses, and the data will be analyzed for statistical significance using the ANOVA statistical test.

The basic experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Example 11

Establishing the parameters affecting amplicon packaging, transport, and expression

The greatest therapeutic value of topical HSV-based amplicon DNA for the treatment of herpes involves (1) packaging of HSV-based amplicon DNA into HSV virions at the lesion site, (2) the spread of HSV-based amplicon DNA into the nervous system and 3) transgene expression in neural cells. While IFN- γ may have antiviral actions at the lesion site, the greatest therapeutic potential of IFN- γ is to facilitate the transition from the productive infection to the latent HSV infection in neurons. Accordingly, this experimental series demonstrates that topically applied HSV-base amplicon DNA is packaged *in situ* into HSV virions, then transported along peripheral nerves, and expressed in the nervous system.

The lips of cotton rats and mice will be inoculated with HSV-1(F). HSV-1(pCMV-LacZ) amplicon DNA will then be applied to the lips of Group 1 animals. Group 2 animals will comprise the control. DNA application will use pre-established conditions for optimal *in situ* packaging of HSV-1(pCMV-LacZ) DNA into HSV-1 virions.

The time course of HSV-1(F) spread from the lip to the nervous system has previously been established using immunohistochemical analysis and infectious virus recovery (titration) assays (data not shown). 15 animals from each group will be sacrificed at 4, 5, 6, 7, 14, and 30 days postinfection. At each time point, 5 animals from group 1 and 3 animals from group 2 (controls) will be perfused with 4% para-formaldehyde in phosphate buffer. The trigeminal ganglia and brains will be removed

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and placed in an 18% sucrose/phosphate buffer solution overnight at 4°C. Frozen sections of 5 µm will be made using a cryostat, and melted onto charged microscope slides. The sections will be incubated with monoclonal antibodies (mAb) specific for β-gal (Boehringer Mannheim), and the HSV-1 immediate early gene, ICP4 (ABI). Primary antibody binding will be visualized with secondary antibodies conjugated to either Cy3 (red fluorescence) or Cy2 (green fluorescence). ICP4 positive- (red), β-gal positive- (green) and double positive cells will be visualized using fluorescence and light microscopy. Cells infected by HSV-1 virions with HSV-based amplicon DNA will be positive for only β-gal (green staining). Cells infected by HSV-1 virions with HSV-1 DNA will be positive for only ICP4 (red staining). Finally, cells infected with both types of HSV-1 virions will be positive for both β-gal and ICP4 (red-, green-, and orange staining). With these data we will determine if HSV-1 virions containing HSV-based amplicon DNA and HSV-1 virions containing HSV-1(F) DNA are transported to the same or different cells in the nervous system.

In addition, 10 animals in group 1 and 8 animals in group 2 will be sacrificed at each time point by CO₂ inhalation to remove the trigeminal ganglia and the brainstem-cerebellum region of the brains. β-gal activity levels will be measured in the tissue homogenate supernatants from 5 animals in group 1 and 3 animals in group 2 per time point as described above. Infectious virus titers at each time point will be measured in the tissue homogenate supernatants from the other 5 animals in each group. The titers of infectious HSV-1 will be plotted against time postinfection. With these data we will establish: 1) the time courses for HSV-1 infection and β-gal transgene expression in the nervous system; 2) whether transgene expression is simultaneously turned off with the termination of HSV-1(F) DNA replication, and; 3) if the time course of HSV infection in the nervous system is altered by the presence of HSV-1 virions with amplicon DNA.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations:

- 1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models;
- 2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models;

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- 3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models; and
- 4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

In the herpes genitalis models, the above-described analysis will be done with the lumbosacral dorsal root ganglia, spinal cords, and brainstems. Similar studies will demonstrate transgene (β -gal and IFN- γ) expression during HSV reactivation.

Example 12

HSV-2(pCMV) DNA is packaged *in situ* and transported to neural sites

Eight cotton rats were inoculated with HSV-2 on their bottom lip. Six hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μ l of HSV-2(pCMV) amplicon DNA (5 μ g) mixed with PEI (9 equivalents) was carefully placed on the inoculation site, using a pipettor. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Four rats were sacrificed on day 5, and 4 rats were sacrificed on day 6. A careful examination of the inoculation sites indicated that 6 of the animals showed no signs of lip lesions at the time of sacrifice. The HSV-2(pCMV) vector does not express IFN- γ or any transgene. Thus, the low incidence of lesion formation in this experiment results because HSV-2(pCMV) DNA competed with infectious HSV-2 DNA for packaging. This effectively reduces the titer of infectious HSV-2 and reduces the likelihood of overt lesions.

DNA was isolated from the trigeminal ganglia (peripheral nervous system) and specific brain regions (brainstem & cerebellum) of all animals and tested for the presence of the β -lactamase (ampicillin resistance) gene by PCR. Three of the animals had detectable β -lactamase DNA in brain and/or trigeminal ganglia. Detection of the β -lactamase gene in the brain and trigeminal ganglia of some HSV-2(pCMV)-treated animals indicates that the amplicon DNA can be packaged *in situ* and transported into the nervous system.

Example 13**Topical application of HSV-2(pCMV) DNA inhibits lesion formation**

Seven cotton rats were inoculated with HSV-2 on their bottom lip. Sixteen hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μ l of HSV-2(pCMV) amplicon DNA (5 μ g) mixed with PEI (9 equivalents) was carefully placed on the inoculation site of four of the rats. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Lip lesion formation for each rat was photographed daily for 5 days. As expected, by day five, the vehicle-treated rats had large herpetic lesions. In contrast, the amplicon-treated rats did not exhibit any obvious lesions.

Figure 12 illustrates this dramatic reduction in lesion formation. The top panels show two of the HSV-2-inoculated rats treated with vehicle only. The bottom panels show two of the HSV-2 inoculated rats treated topically with HSV-2(pCMV) DNA.

All animals were sacrificed on day 5 and DNA was isolated from neural tissues as above. The β -lactamase gene was not detected in the DNA's from HSV-2(pCMV)-treated animals in this experiment.

This specification is most thoroughly understood in light of the teachings of the references cited herein, all of which are hereby incorporated by reference in their entirety.

The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan recognizes that many other embodiments are encompassed by the claimed invention and that it is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

-35-

We claim:

1. **An *in situ* method for packaging DNA into a viral particle comprising applying at least one unpackaged DNA construct to the site of an active viral infection in an organism, wherein at least one applied DNA construct is packaged into viral particles.**
2. The method of claim 1, wherein the viral infection is a Herpesvirus infection.
3. The method of claim 2, wherein at least one DNA construct is an amplicon.
4. The method of claim 3, wherein the amplicon comprises sequences compatible with packaging into an HSV virion.
5. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-1.
6. The method of claim 5, wherein the amplicon further comprises an HSV ori sequence.
7. The method of claim 6, wherein the HSV ori sequence is an HSV-1 ori_s sequence.
8. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-2.
9. The method of claim 8, wherein the amplicon further comprises an HSV ori sequence.
10. The method of claim 9, wherein the HSV ori sequence is a HSV-2 ori_s sequence.
11. **A method of treating a viral infection comprising the steps of:**
 - 1) **applying a composition comprising at least one unpackaged DNA construct to a site of viral infection in an organism, and**
 - 2) **packaging at least one applied DNA construct *in situ*.**
12. The method of claim 11, wherein the viral infection is a Herpesvirus infection.
13. The method of claim 12, wherein the Herpesvirus is HSV-1 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-1.
14. The method of claim 13, wherein at least one unpackaged DNA further comprises an HSV ori sequence.

-36-

15. The method of claim 12, wherein the Herpesvirus is HSV-2 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-2.
16. The method of claim 15, wherein the at least one unpackaged DNA further comprises an HSV ori sequence.
17. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.
18. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.
19. The method of claim 11, wherein at least one applied DNA construct is capable of replicating in a host organism in the presence of an active viral infection.
20. The method of claim 11, wherein the composition is applied topically.
21. The method of claim 11, wherein the organism is human.
22. **A method of treating a viral infection comprising the steps of:**
 - 1) applying a composition comprising one or more unpackaged amplicons to a site of viral infection in an organism,**
wherein one or more of the amplicons encode at least one anti-viral compound, and
 - 2) expressing at least one of the encoded anti-viral compound in the organism.**
23. The method of claim 22, wherein the viral infection is a Herpesvirus infection.
24. The method of claim 23, wherein the Herpesvirus is HSV-1.
25. The method of claim 24, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-1.
26. The method of claim 25, wherein one or more amplicons further comprise a HSV ori sequence.
27. The method of claim 26, wherein the HSV ori sequence is a HSV-1 ori_s sequence.
28. The method of claim 23 wherein the Herpesvirus is HSV-2.

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29. The method of claim 28, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-2.
30. The method of claim 29, wherein one or more amplicons further comprises a HSV ori sequence.
31. The method of claim 30, wherein the HSV ori sequence is a HSV-1 ori_s sequence.
32. The method of claim 22, wherein one or more amplicons are packaged *in situ*.
33. The method of claim 22, wherein at least one anti-viral compound is a cytokine.
34. The method of claim 33, wherein the cytokine is an interferon.
35. The method of claim 34, wherein the cytokine is interferon gamma (IFN-γ).
36. The method of claim 35, wherein the interferon gamma is human interferon gamma.
37. The method of claim 22, wherein at least one antiviral compound is expressed during active infection.
38. The method of claim 22, wherein expression of at least one antiviral compound is constitutive.
39. The method of claim 22, wherein expression of the antiviral compound is stress-inducible.
40. The method of claim 22, wherein at least one antiviral compound is expressed in epithelial cells.
41. The method of claim 22, wherein at least one antiviral compound is expressed in neuronal cells.
42. The method of claim 22, wherein expression of at least one anti-viral compound is directed by a fos promoter.
43. The method of claim 22, wherein expression of at least one anti-viral compound is directed is directed by a CMV immediate early gene promotor.
44. The method of claim 22, wherein the organism is human.
45. The method of claim 22, wherein the organism is a non-human animal.
46. The method of claim 22, wherein the composition is applied topically.

-38-

47. The method claim 23, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.

48. The method claim 23, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.

49. **A composition comprising one or more amplicons capable of directing the inducible expression of at least one anti-viral compound in neuronal cells, wherein the expression of the at least one anti-viral compound in neuronal cells decreases the frequency or severity of HSV reactivation in an organism.**

50. The composition of claim 49, wherein the promoter is stress-inducible.

51. The composition of claim 49, wherein the promoter is a fos promoter.

52. The composition of claim 49, wherein the anti-viral compound is a cytokine.

53. The composition of claim 52, wherein the cytokine is an interferon.

54. The composition of claim 53, wherein the interferon is IFN- γ .

55. The composition of claim 54, wherein the interferon is human IFN- γ .

56. The composition of claim 49, wherein one or more amplicons comprises an HSV a' sequence and an HSV ori sequence.

57. The composition of claim 56, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-1 infection.

58. The composition of claim 57, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-2 infection.

59. The composition of claim 57, further comprising a carrier.

60. The composition of claim 49, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.

61. The composition of claim 49, wherein the organism is a human.

62. **A method of reducing the frequency or severity of HSV recurrences in an organism comprising administering the composition of claim 49.**

63. The method of claim 62, wherein the composition is applied topically to the site of an active HSV infection.

64. The method of claim 63, wherein the composition is applied during the prodrome stage.

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65. The method of claim 63, wherein the composition is applied during a vesicular stage.
66. **A composition for treating HSV infections comprising one or more amplicons programmed to express:**
- 1) at least one anti-viral compound in HSV infected epithelial cells, and**
 - 2) at least one anti-viral compound in HSV infected neuronal cells, wherein amplicon-directed expression of at least one anti-viral compound in HSV infected neuronal cells is inducible.**
67. The composition of claim 66, wherein at least one anti-viral compound is a cytokine.
68. The composition of claim 67, wherein the cytokine is an interferon.
69. The composition of claim 68, wherein the interferon is IFN- γ .
70. The composition of claim 69, wherein the interferon is human IFN- γ .
71. The composition of claim 66, wherein neuronal expression is stress inducible.
72. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a fos promoter.
73. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a constitutively active promoter.
74. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by the CMV immediate early gene promoter.
75. The composition of claim 66, wherein one or more amplicons comprise unpackaged DNA.
76. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-1.
77. The composition of claim 76, wherein the one or more amplicons further comprise an HSV ori sequence.
78. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-2.
79. The composition of claim 78, wherein the one or more amplicons further comprise an HSV ori sequence.

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- 80. The composition of claim 66, further comprising a carrier.
- 81. The composition of claim 80, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.
- 82. **A method for treating HSV infection comprising administering the composition of claim 66 to an organism.**
- 83. The method of claim 82, wherein the organism is human.
- 84. The method of claim 82, wherein the organism is a non-human animal.
- 85. The method of claim 82, wherein the composition is applied topically.
- 86. The method claim 82, wherein the composition is applied during the prodrome stage.
- 87. The method claim 82, wherein the composition is applied during a vesicular stage.

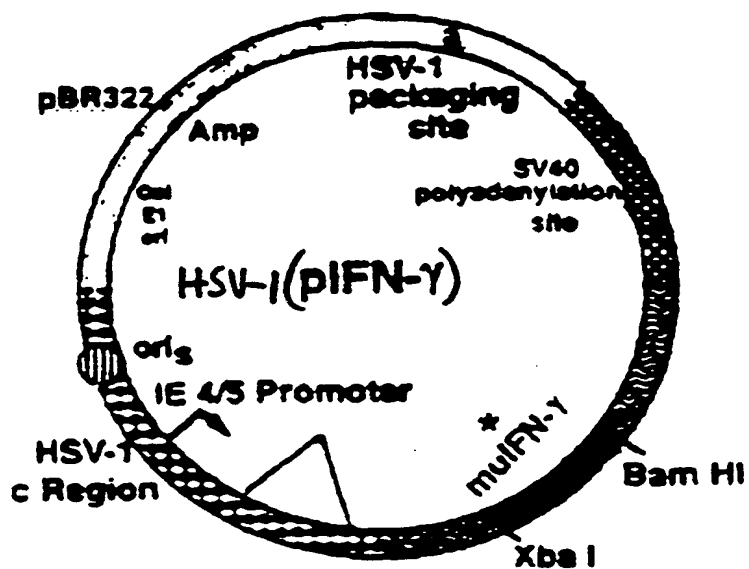


Figure 1

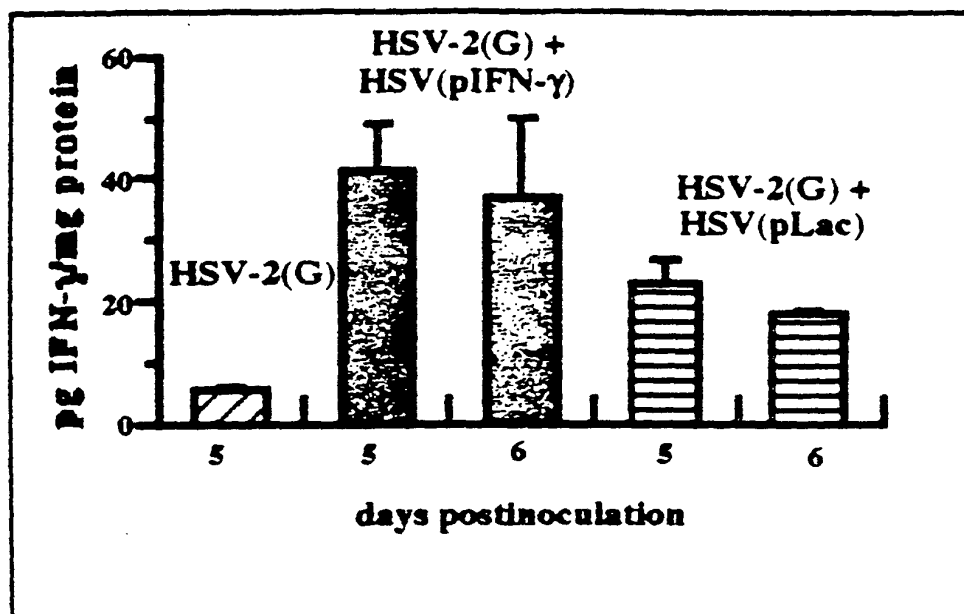


Figure 2

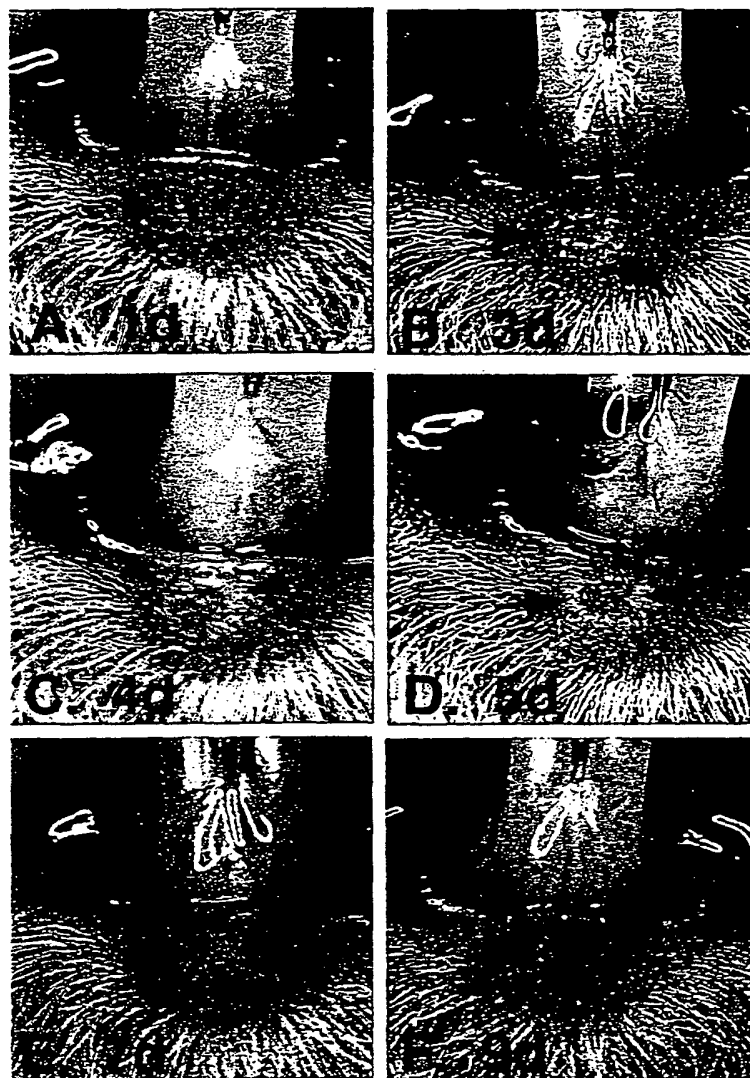


Figure 3

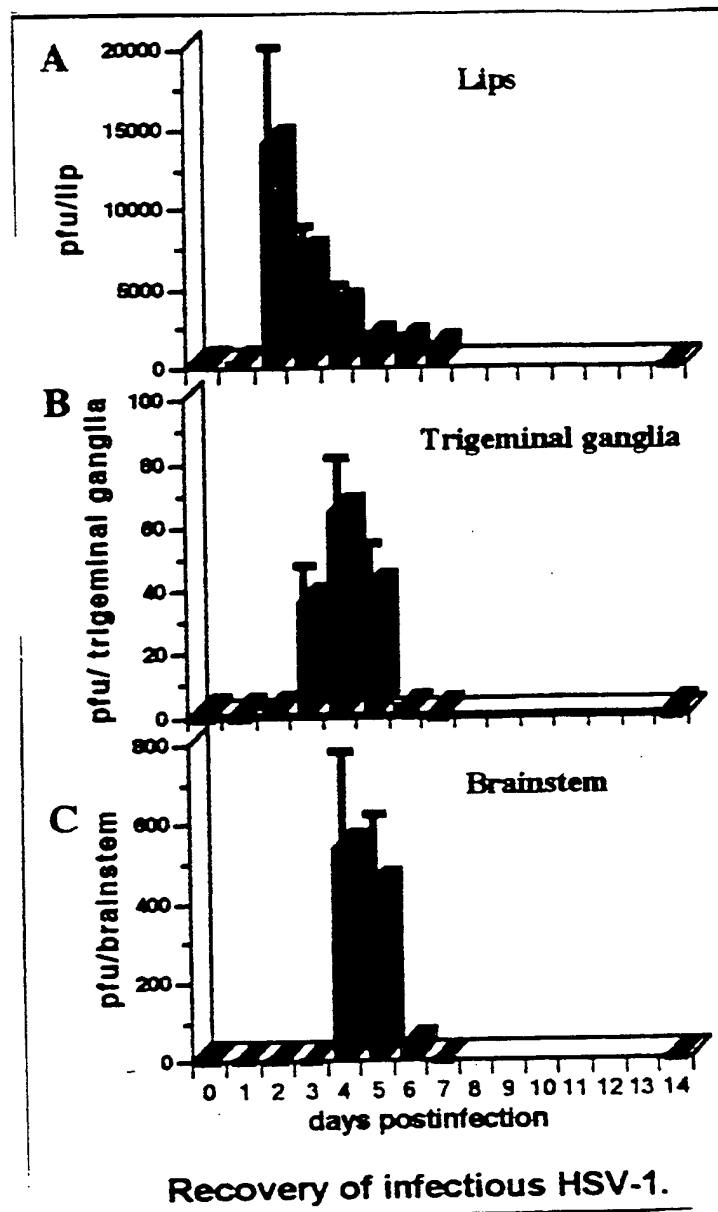


Figure 4

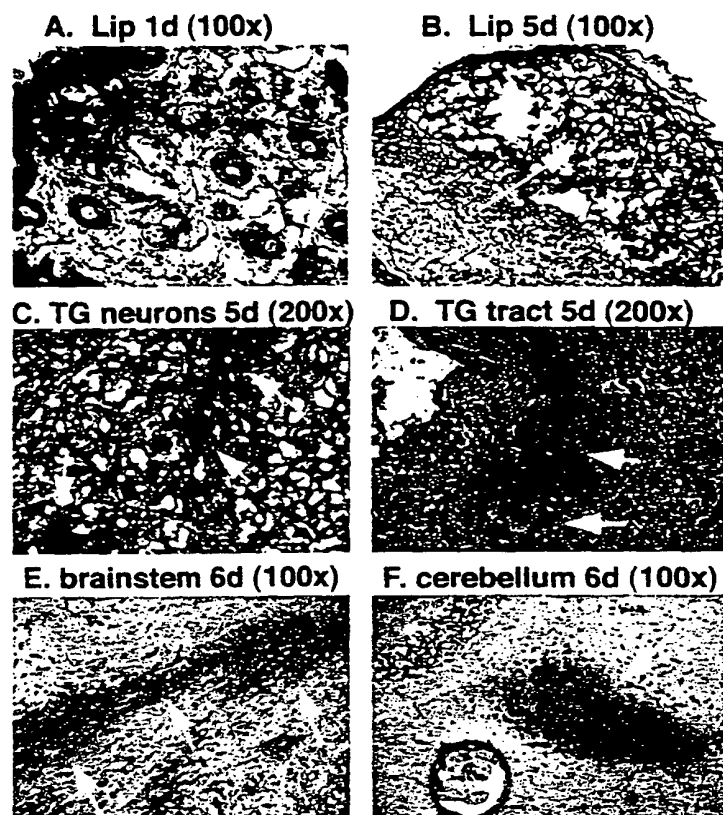


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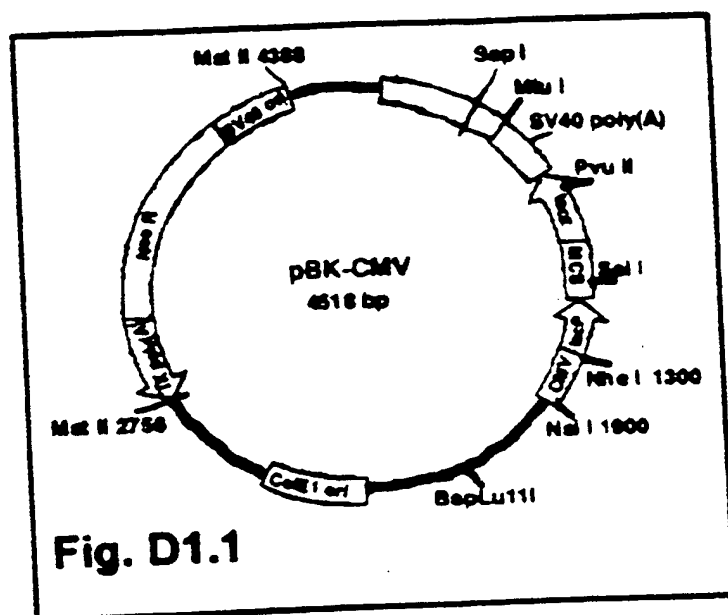


Figure 6

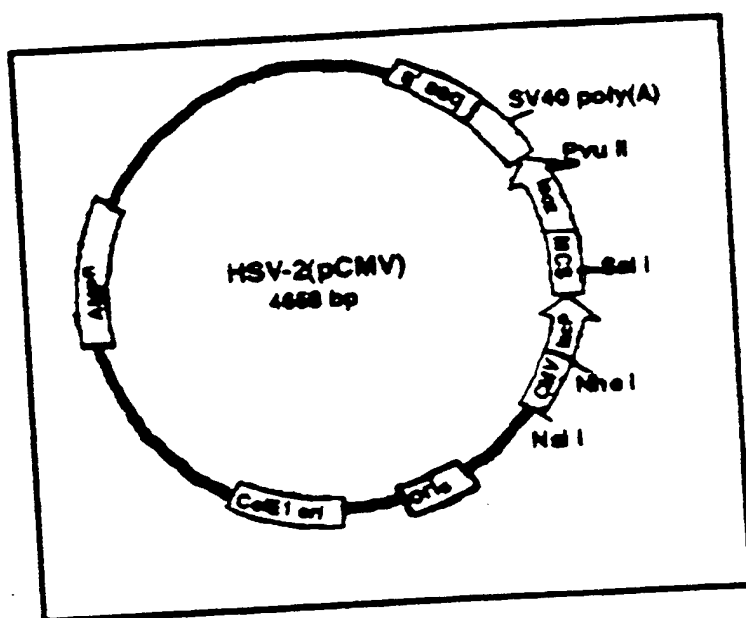


Figure 7

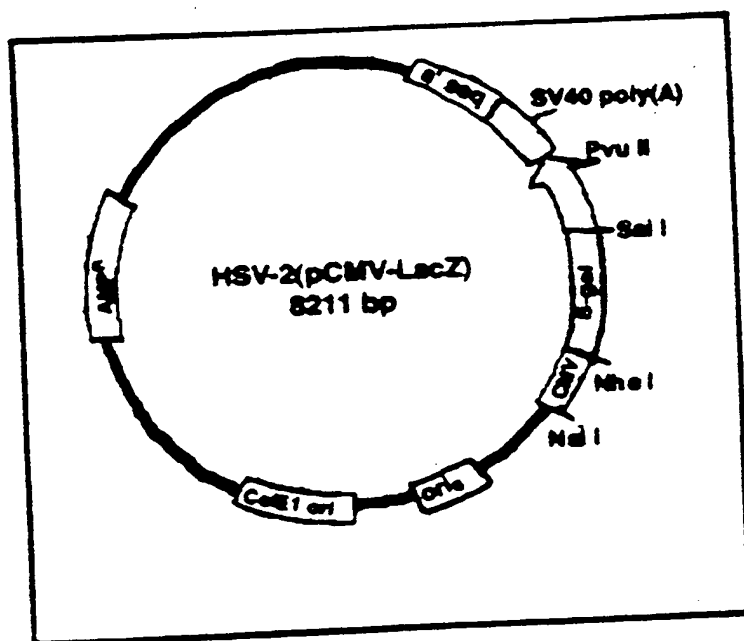


Figure 8

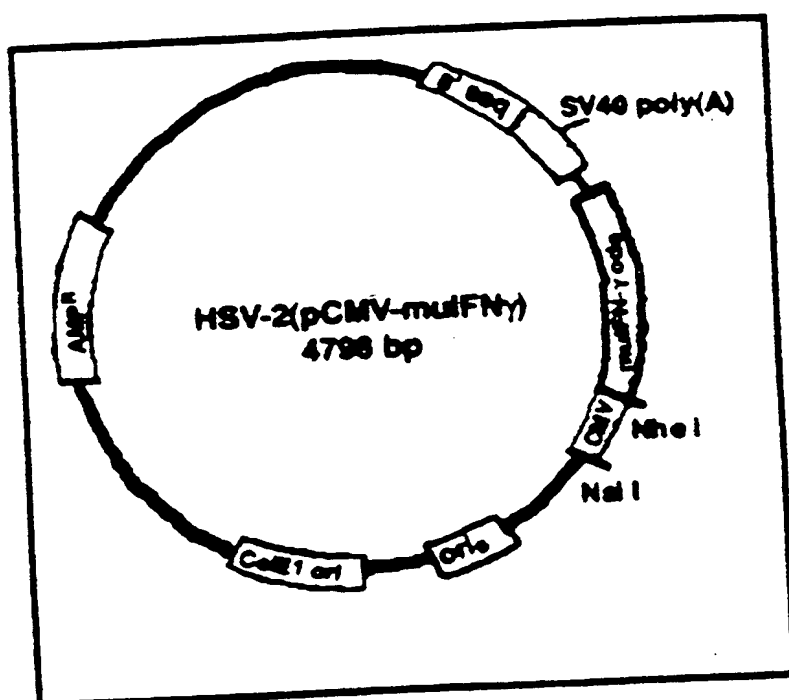


Figure 9

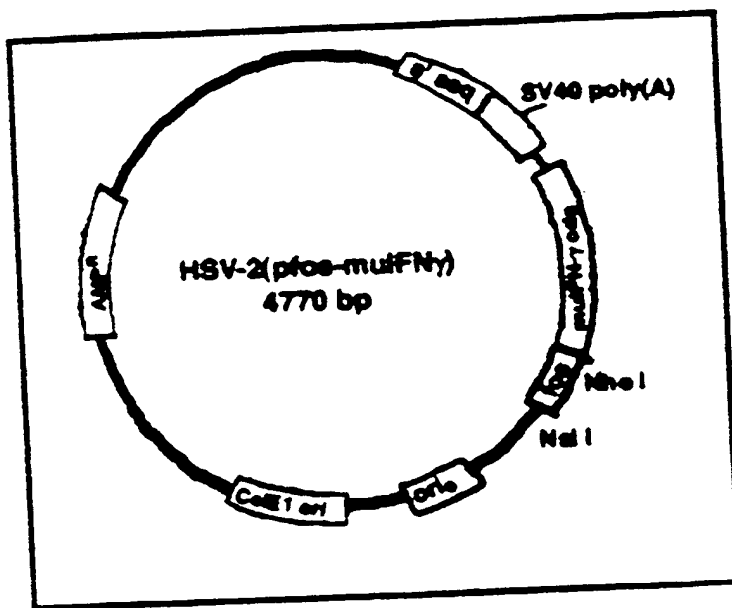


Figure 10

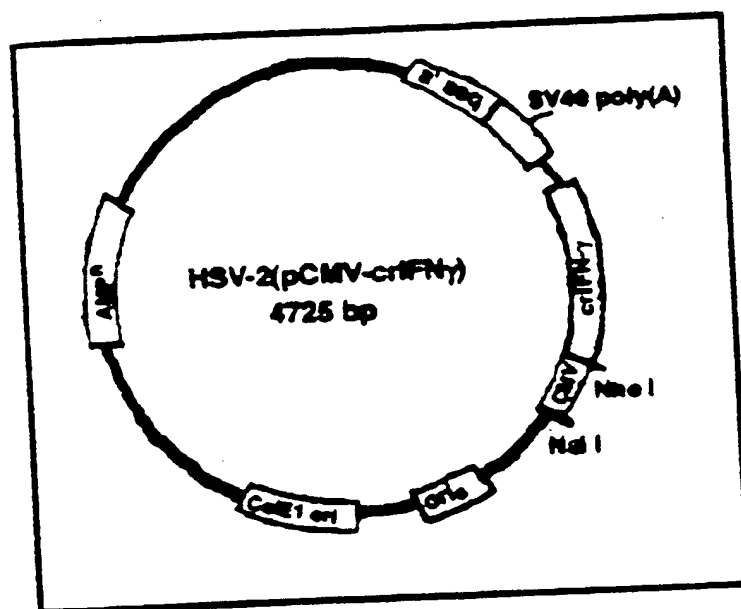


Figure 11

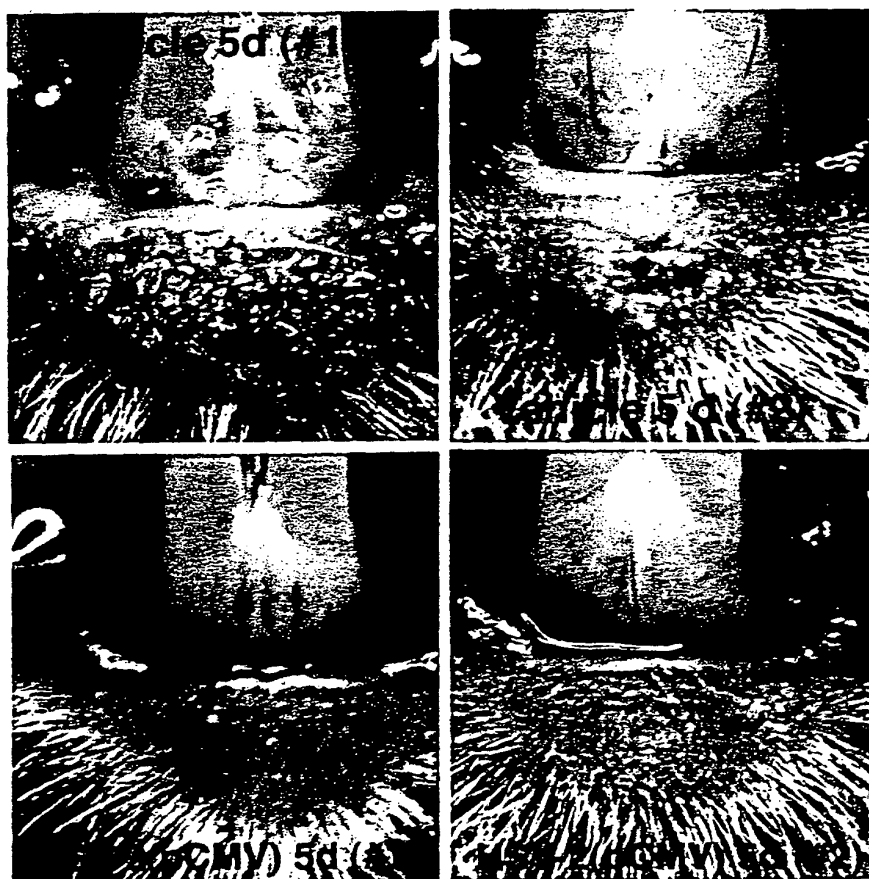


Figure 12

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05594

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 A61K48/00 A61K38/21 A61K47/20 A61K47/30
A61P31/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. LEWANDOWSKI ET AL.: "Evidence that deficient IFN gamm production is a biological basis of herpes simplex virus type 2 neurovirulence" J. NEUROIMMUNOL., vol. 81, 1998, pages 66-75, XP000929623 cited in the application the whole document	49, 52-58, 61, 66-70, 73,76-79
A	B. LU ET AL., : "Topical application of viral vectors for epidermal gene transfer" J. INVEST. DERMATOL., vol. 108, no. 5, 1997, pages 803-808, XP000929624 page 805 -page 808 --- -/-	11-48



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

4 August 2000

Date of mailing of the international search report

21/08/2000

Name and mailing address of the ISA

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Authorized officer

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05594

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. STARR ET AL.: "Long-term persistence of defective HSV-1 vectors in the rat brain is demonstrated by reactivation of vector gene expression" GENE THERAPY, vol. 3, 1996, pages 615-623, XP000929634 the whole document	
A	WO 99 08713 A (UAB RESEARCH FOUNDATION) 25 February 1999 (1999-02-25) the whole document	11-48

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 00/05594

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9908713 A	25-02-1999	AU 8780798 A EP 1015035 A	08-03-1999 05-07-2000
<hr/>			

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 4995.52-304	FOR FURTHER ACTION		see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US 00/ 05594	International filing date (day/month/year) 03/03/2000	(Earliest) Priority Date (day/month/year) 03/03/1999	
Applicant LEWANDOWSKI, Gail et al.			

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ **None of the figures.**

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 00/05594

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 A61K48/00 A61K38/21 A61K47/20 A61K47/30
A61P31/22

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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search

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Name and mailing address of the ISA

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 Fax: (+31-70) 340-3016

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Skelly, J

INTERNATIONAL SEARCH REPORT

International Application No.

Patent No. 00/05594

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 99 08713 A (UAB RESEARCH FOUNDATION) 25 February 1999 (1999-02-25) the whole document -----	11-48

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/05594

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9908713 A	25-02-1999	AU 8780798 A EP 1015035 A	08-03-1999 05-07-2000

09/ 914,840

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
8 September 2000 (08.09.2000)

PCT

(10) International Publication Number
WO 00/52185 A1(51) International Patent Classification⁷: C12N 15/86.
A61K 48/00, 38/21, 47/20, 47/30, A61P 31/22

(74) Agents: GARRETT, Arthur, S. et al.; Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P., 1300 I Street, N.W., Washington, DC 20005-3315 (US).

(21) International Application Number: PCT/US00/05594

(81) Designated States (*national*): AU, CA, JP, US.

(22) International Filing Date: 3 March 2000 (03.03.2000)

(84) Designated States (*regional*): European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

(25) Filing Language: English

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Published:
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20 June 2002

(71) Applicants and

(72) Inventors: LEWANDOWSKI, Gail [US/US]; 5800 North Kolb, Apt. 12266, Tuscon, AZ 85750 (US). PRINCE, Gregory, A. [US/US]; 14800 Petit Way, Potomac, MD (US). GUZOWSKI, John [US/US]; 5800 North Kolb, Tuscon, AZ 85750 (US).

(15) Information about Correction:
see PCT Gazette No. 25/2002 of 20 June 2002, Section II*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 00/52185 A1

(54) Title: APPLICATION OF DNA VECTORS FOR THE TREATMENT OF VIRAL INFECTION

(57) **Abstract:** The present invention relates to DNA-based constructs and methods for treating viral infections. In a preferred embodiment, an HSV based amplicon is topically applied to the site of an actively replicating HSV infection, initiating a four-tier anti-viral therapy. First, IFN- γ expression from a constitutive promoter exerts an antiviral effect at the site of active infection. Second, replication machinery of the resident virus packages the amplicon into HSV virions, *in situ*, thereby decreasing the infectious titer. Third, HSV virion containing amplicon DNA are transported into the nervous system where IFN- γ expression promotes the establishment of latency. Fourth, as the host is exposed to emotional or physical stressor that reactivate the virus, amplicon-directed IFN- γ is expressed. Increasing titers of IFN- γ enforce viral latency.

-1-

Application of DNA Vectors for the Treatment of Viral Infection

GOVERNMENT INTEREST

This invention was made, in part, with Government support under NIH grant no. RO1-MH58374 awarded by the National Institutes of Health. The U.S. Government may have certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to DNA-based constructs and methods for treating viral infections. The preferred constructs are amplicons containing DNA that can be packaged *in situ* by an active viral infection, preferably a Herpesvirus infection, and more preferably, herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) infections. The amplicons may express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). In a highly preferred embodiment, one or more amplicons express IFN- γ in the epithelial and neural cells of an organism infected with HSV.

The present invention further relates to the use of these constructs for the treatment of viral infections. In a highly preferred embodiment, amplicon DNA is applied topically to a site of active HSV infection and packaged *in situ*. Locally, amplicon-directed cytokine expression reduces the titer of HSV to ameliorate the severity and duration of the active (or productive) infection. Amplicon DNA packaged *in situ* by active HSV is transported to the nervous system. In the nervous system amplicon-directed IFN- γ expression induces the virus to enter a latent phase. The amplicons of the invention are constructed so that environmental stimuli which reactivate HSV also induce the amplicon-directed expression of IFN- γ . The induced IFN- γ maintains or re-establishes HSV latency, thereby eliminating or reducing recurrent infection.

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BACKGROUND

The human Herpesviruses comprise at least eight distinct DNA viruses, including herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and three recently described viruses designated human herpesvirus types 6, 7, and 8. In addition, a large number of Herpesviruses cause disease in non-human hosts including, Equid herpesviruses 1-8; Suid herpesviruses 1 and 2; and Gallid herpes viruses 1-3. Known members of the Herpesviridae family are reviewed in Bernard Roizman, Herpesviridae, Fields Virology, 3d ed., Ch. 71, incorporated herein by reference. All herpes viruses produce both active and latent infections, which permits viral reactivation and recurrent clinical illness throughout the life of the host. The recurrent nature of Herpesvirus infection promotes transmission and contributes to the high prevalence of these viruses.

HSV-1 and HSV-2 (collectively, HSV) have been identified as the causative agents of recurrent oral and genital herpetic lesions. HSV-1 and HSV-2 are highly related and present clinically indistinguishable symptoms. Nevertheless, each exhibits preferential sites of infection such that HSV-1 is primarily responsible for oral-labialis herpes, whereas HSV-2, and to a lesser extent, HSV-1, are responsible for genital and anal herpes.

Clinical manifestations of HSV infection are reviewed in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d ed., Ch. 73, incorporated herein by reference. HSV possesses both a lytic and a latent phase. The lytic phase begins with exposure to HSV at mucosal surfaces or abraded skin. The virus gains entry into epidermal and dermal cells and initiates viral replication.

The active HSV infections of the lytic cycle frequently result in painful vesicles filled with highly infectious fluid appearing on the dermal and mucosal surfaces of the lips, mouth, pharynx, and nasal passages (oral-labial herpes), and genitals, buttocks, thighs and anal regions (genital herpes). Vesicle formation is frequently preceded by prodrome stage, characterized by a tingling or burning sensation. The infection site reddens (macule or erythema stage) and fluid-filled vesicles appear rapidly after the onset of prodrome, within about six hours in the case HSV-1 infection. Within 3 to 4

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days the vesicular fluid becomes pustular due the recruitment of inflammatory cells. On dry, external surfaces, the lesions develop a crusting, scab-like appearance, whereas shallow ulcers are more common on moist mucosal membranes. Untreated, oral infections are generally resolved in 8-10 days, but genital lesions persist for an average of three weeks.

Following primary infection, the virus travels to the neuronal nuclei in the dorsal root ganglia of the sacral, vagal, and/or trigeminal nerves where it remains latent for indefinite periods. While in a latent state, HSV does not produce infectious virus, does not cause disease, and is not transmissible. However, when HSV reactivates, it is transmissible, even if the episode is subclinical or asymptomatic. (Barton et al., *Int. J. STD. AIDS* 7:229-32 (1996)). Reactivation may be triggered by emotional stress and a variety of environmental factors including, UV light, trauma, heat, fever, colds, fatigue, menses, and certain foods, and usually results in the appearance of new lesions at or near the site of initial infection.

The frequency of reactivation is patient-dependent but may be quite high. About 23% of those infected orally with HSV-1 experience 2 or more recurrences per year. Genital (primarily HSV-2) outbreaks follow a somewhat different pattern. About 500,000 to one million new cases of genital herpes occur in the United States alone. Although only 20-25% of those infected with genital herpes are aware of their condition, more than half of the patients who experience recurrences report at least one episode per month. (Hamuy, R. and B. Berman. 1998. Treatment of Herpes simplex virus infections with topical antiviral agents. *Eur. J. Dermatol.* 8:310-319).

HSV-1 and HSV-2 are easily transmissible and a large proportion of the population has been infected. Worldwide, 60 to 90% of adults have been infected with HSV-1, and nearly 25% have been infected with HSV-2. HSV is not commonly considered a critical health concern among immunocompetent adults, but for people who suffer frequent oral or genital recurrences, herpes can greatly decrease the quality of life. Although frequently painful, lesions generally heal completely. However, severe or recurrent outbreaks can result in scarring, and infections of the eye may result in corneal blindness. (John Manos, DNA Viruses 1: Herpesviruses, Microbiology and infectious

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diseases). Damage to peripheral nerves may result in facial paralysis or Bell's palsy, and movement disorders in children. (Billue, Nurse. Pract. 22:88, 97-100, and 102-105 (1997); Hargrave et al., Dev. Med. Child. Neurol. 40:640-42 (1998)).

In immunocompromised patients, including patients receiving immunosuppressant drugs in association with organ or bone marrow transplants and those with the acquired immunodeficiency syndrome (AIDS), lesions may be chronic, progressive and disabling. Similarly, immunological changes occurring during pregnancy place both the mother and fetus at a high risk of severe infection. Active infection during pregnancy has been associated with spontaneous abortion and occasionally with life-threatening systemic maternal infection. (Fields, Bernard, et al., Fields Virology, Herpes simplex viruses, vol. 2, Ch. 73).

Although the spread of HSV through the nervous system is principally confined to peripheral nerves, HSV is implicated in a particularly lethal form of viral encephalitis. HSV encephalitis is the most common viral infection of the central nervous system, accounting for 10-20% of all isolated viral CNS infections in the United States. Left untreated, HSV encephalitis has a mortality rate of about 70%. Less well recognized CNS associations include mood and behavioral dysfunctions, Alzheimer's disease, and profound global amnesia. (Becker, Y., Virus Genes 10:217-226 (1995); Caparros-Lefebvre, D., et al, J. Neurol. 243:248-256 (1996); Itzhaki, R. F., et al., Lancet 352:238 (1998); Lin et al., Mol. Chem. Neuropathol. 28:135-41 (1996); Kimura, S., et al., Intern. Med. 34:131-133 (1995); Wilson, B. A., et al., J. Clin. Exp. Neuropsychol. 17:668-681 (1995)).

In accord with these CNS disturbances, studies in mice and cotton rats strongly argue that HSV invades the brain during primary ocular HSV infection (Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994) and Lewandowski et al., Proc. Natl. Acad. Sci. USA 90:2005-2009 (1993)), and primary HSV labialis infection (Lewandowski et al. manuscript in preparation). Moreover, because HSV moves along the nerves in both retrograde and anterograde directions, HSV has the potential to spread further into the nervous system with each recurrent episode. Consequently, even if initially limited to the peripheral nervous system, HSV has the potential of invading the brain every time

latency is broken. Notably, no current herpes therapy is designed to keep HSV in a latent state.

Currently, nucleoside analogs have proven somewhat successful at inhibiting HSV replication in the active stages of infection. These compounds include competitive inhibitors of HSV DNA polymerase: the pyrophosphate, Foscarnet, and the acyclic nucleoside phosphonate, cidofovir (De Clercq, E., *Intervirology* 40:295-303 (1997); Martinez, C. M. et al., *Pharmacotherapy* 31:1519-1521 (1997); Oram, R. J., et al, *Pediatr. Infect. Dis. J.* 17:652-653 (1998)). However, nearly all current herpes therapies rely on acyclic nucleoside analogs such as acyclovir (ACV), valacyclovir, famciclovir, penciclovir (PCV), and ganciclovir (Erlach, K., et al., *J. Med.* 166:211-215 (1997); Oram, R. J., et al., *Pediatr. Infect. Dis. J.* 17:652-653 (1998); Wutzler, P., *Intervirology* 40:343-356 (1997)). These compounds are selectively monophosphorylated by the herpesvirus thymidine kinase, but not by the cellular enzyme. The monophosphate nucleotide analog is then further phosphorylated by cellular kinases to the di- and triphosphate forms. The triphosphorylated nucleotide analog interacts with and inhibits the viral DNA polymerase.

In some patients, oral or intravenous administration of nucleoside analog compounds result in a faster healing of lesions, a shortened duration of virus shedding, and a faster resolution of symptoms. In addition, suppressive (daily) oral acyclovir, valacyclovir, or famciclovir may reduce the severity and frequency of recurrent episodes in some patients (Erlach, K. S., *West. J. Med.* 166:211-215 (1997); Faro, S., *Infect. Dis. Obstet. Gynecol.* 6:38-43, (1998)). However, it is likely that suppressive dosage schedules will increase the inherent risk of drug-resistant HSV strains (Reusser, P., *J. Hosp. Infect.* 33:235-248 (1996)). Indeed, acyclovir-resistant and Foscarnet-resistant HSVs have already been described. (Erlach, K. S., *West. J. Med.* 166:211-215 (1997)).

HSV infection can also be treated by topical administration of anti-herpetic drugs. (Hamuy, R. et al., *Eur. J. Dermatol.* 8:310-319 (1998)). However, topical therapies have at least two challenges. First, the drug must be specially formulated to penetrate the stratum corneum barrier in the skin. Currently, the approved topical formulations include ACV in polyethylene glycol, and ACV or PCV in an aqueous cream. Unfortunately, transdermal delivery in these approved vehicles is very poor and slow. (Freeman, D.

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J., et al., Antimicrob. Agents. Chemother. 29:730-732; Spruance, S. L., J. Infect. Dis. 146:85-90 (1982)). Secondly, topical application must begin during the brief "prodrome" stage, concurrent with the initiation of HSV replication. Nevertheless, patient-initiated applications frequently do not begin until the lesion becomes visible and the HSV titer is already high.

Consequently, there remains a need in the art for a safe and efficacious treatment for HSV. In particular, there remains a need for treatment which will prevent recurrent outbreaks of active infection, reduce viral transmission, and limit the propagation of infected tissues in a host.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing a novel system for treating viral infection, preferably, Herpesvirus infections, and more preferably, HSV-1 and HSV-2 infections, using DNA-based constructs. In one embodiment, the constructs are capable of being packaged *in situ* by an active (replicating) viral infection. In another embodiment, the constructs are amplicons programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). In a preferred embodiment, the constructs are both capable of being packaged *in situ* by a replicating virus and expressing anti-viral compounds.

In a highly preferred embodiment, unpackaged amplicon DNA is applied to the site of an active HSV infection and packaged *in situ*. Consequently, amplicon DNA is incorporated into nascent virus particles in lieu of viral genomes. By reducing the concentration of capsids available to the replicating virus, *in situ* packaging of the amplicon attenuates the infection, decreases infectivity and hastens lesion healing. Concurrently, amplicon-directed IFN- γ expression interferes with viral replication, attenuates the on-going infection, and accelerates the onset of latency. In a highly preferred embodiment, amplicon DNA also remains resident in neuronal cells, the same as, or near to those which harbor latent HSV. The same environmental signals which reactivate the latent HSV virus also induce the amplicon-directed expression of IFN- γ . Consequently, viral latency is maintained or quickly re-established.

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BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 HSV-1(pIFN- γ) is schematically depicted in Figure 1. HSV-1 sequences include an a' region packaging site; an HSV-1 origin of DNA replication (ori_s); a transcriptional unit (HSV-1 c Region) containing an HSV-1 immediate early gene promoter (IE 4/5 promoter) and intervening sequences following the promoter. Non-HSV features include a pBR322 backbone containing a prokaryotic origin of replication (Col E1 Ori) and the ampicillin resistance conferring sequence (Amp); the SV40 polyadenylation site; and a mouse interferon gamma coding sequence (muIFN- γ).
- Figure 2 Figure 2 compares the total IFN- γ production in the brains of mice infected with the HSV-2(G) strain of HSV-2 (column 1); mice co-infected with HSV-2(G) and HSV(pIFN- γ) (columns 2-3) DNA; and mice co-infected with HSV-2(G) and control vector DNA, HSV(pLac) (columns 5-6). Constructs HSV-1(pIFN- γ) and HSV-1(pLac) are designated HSV(pIFN- γ) and HSV(pLac), respectively, in Lewandowski et al., J. Neuroimmunol. 55:23-34 (1994).
- Figure 3 Color photographs of HSV-1 induced herpes labialis in the cotton rat model.
- Figure 4 HSV-1 titers in lips, trigeminal ganglia and brainstem of HSV-1 infected cotton rats.
- Figure 5 Color photograph of immunohistochemically stained samples from cotton rats infected with HSV-1. Dark stain indicates HSV-1 protein detection.
- Figure 6 Schematic representation of commercially available pBK-CMV phagemid.
- Figure 7 Schematic representation of amplicon HSV-2(pCMV).
- Figure 8 Schematic representation of amplicon HSV-2(pCMV-LacZ).
- Figure 9 Schematic representation of amplicon HSV-2(pCMV-muIFN- γ).
- Figure 10 Schematic representation of amplicon HSV-2(pfos-muIFN- γ).
- Figure 11 Schematic representation of amplicon HSV-2(pCMV-crIFN- γ).
- Figure 12 Color photographs illustrating the reduction in oral lesion formation upon topical application of HSV-2(pCMV) in the cotton rat model.

DETAILED DESCRIPTION OF THE INVENTION

While not intending to be bound to any one theory in the practice of this invention, the expression vectors, treatment systems, and methods described herein are believed to rely on well known properties of viral replication known to be shared among many viral families of large DNA viruses. Although one of skill in the art will recognize that the present invention may be used to treat a host of infectious viral diseases, in the interests of brevity, the Examples 1-13 are confined to Herpesvirus, in particular, HSV-1 and HSV-2. Consequently, this invention may be best understood in light of the current understanding HSV life-cycle. Details of HSV biology may be found in Bernard Roizman and Amy E. Sears, Herpes Simplex Viruses and Their Replication, Fields Virology Ch. 72 (3d. Ed., B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated by reference), and in the other referenced cited herein.

The HSV virion consists of four physical elements: 1) an electron-opaque core, containing approximately 150 kbp of double-stranded, predominantly linear DNA; 2) an icosadeltahedral capsid surrounding the core; 3) an amorphous tegument surrounding the capsid; and 4) a glycoprotein-studded outer lipid envelope.

In clinical infections, the virus attaches to cell-surface receptors, usually at a site of dermal or mucosal abrasion. The viral envelope fuses with the plasma membrane of epithelial cells and the de-enveloped capsid enters the cytoplasm. The capsid is transported to the nuclear pores and the DNA is released into the nucleus where it circularizes. Viral transcription, and capsid assembly take place in the nucleus. New copies of viral DNA are produced by rolling-circle replication, cleaved into monomers and packaged into preformed capsids. Tegument proteins and the lipid envelope are added and mature virus is exported through the endoplasmic reticulum. This active infection process is usually accompanied by the localized cell necrosis, inflammation, and vesicle formation characteristic of clinical lesions. Ultimately, however, the virus is cleared from the inoculation site.

In addition, the HSV viruses are highly neurotropic and can spread from the inoculation site to peripheral sensory nerves. Retrograde transport delivers the viral capsids up the nerve to the dorsal root ganglia. Again, the DNA is released through the

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nuclear pores into the nucleus and circularizes. However, unlike the situation in epithelial cells, the HSV DNA may remain latent, exhibiting little or no detectable transcription or replication. Nevertheless, under certain conditions, this apparently quiescent episomal construct can be reactivated. The DNA replicates and new infectious particles are transported back down the peripheral sensory nerves by anterograde transport. Upon reaching the synapses, infectious particles attach to epithelial cells at or near the initial site of inoculation and re-establish an active mucocutaneous infection.

Both the clearance of mucocutaneous infection and the reactivation of latent virus have long been associated with the immune status of the host. (See e.g., J. Lindsay Whitton and Michael B.A. Oldstone Immune Response to Viruses, Fields Virology Ch. 10, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996) (incorporated herein by reference)). This observation has spawned numerous searches for immunological components involved in virulence. Interferons alpha, beta and gamma have been suggested as treatments for HSV infection. (Hamuy et al., Eur. J. Dermatol. 8:310-9 (1998); Smith et al., Virol. 202:76-88 (1994)).

Recently, it has been discovered that interferons, especially IFN- γ , have a potent inhibitory effect on both HSV replication and transcription termination. (Feduchi, E. et al., Virol. 180:822-825 (1991); Komatsu, T., et al., J. Neuroimmunol. 68:1010-108 (1996); Lewandowski, G., et al., J. Neuroimmunol. 81:58-65 (1998)); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998); Schijns, V., et al., J. Neuroimmunol. 28:1-7 (1990); Svennerholm, B., et al., Arch. Virol. 104:153-156 (1989)). Consequently, the production of one or more interferons at the site of infection would hasten the resolution of an active infection. More importantly, the production of one or more interferons at the site of a latent virus would serve to keep the virus in the latent phase, eliminating or reducing the frequency and/or severity of recurrent episodes and limiting the spread of the virus in the host.

The present invention thus encompasses means for delivering antiviral compounds, preferably cytokines, most preferably IFN- γ , to the site of active and/or latent HSV infection. Although any delivery mechanism is acceptable, it is preferred that

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the antiviral compound is a protein expressed at or near the desired site of action. Acceptable delivery systems include amplicons, viruses, defective viruses or defective interfering viruses (DI particles), or combinations thereof, which have been engineered to express IFN- γ , and/or other efficacious compounds. (See, e.g., Donald M. Coen and Robert F. Ramig, Viral Genetics, Fields Virology Ch. 5, (Third Ed. B.N. Fields et al., Eds., Lippincott-Raven 1996); Ho, Methods in Cell Biology 43:191-210 (1994); and U.S. Patent No. 5,672,344, each of which is incorporated herein by reference.)) In a preferred embodiment, the delivery system is an amplicon, more preferably, an amplicon based on HSV-1 or HSV-2 elements.

Specific details of constructing and propagating amplicons based on HSV sequences are available in U.S. Patent Nos. 5,501,979; 5,661,033; 5,672,344; 5,599,691; and in Angela P. Dyer and Frank Tufaro, Herpes Simplex Virus Vectors for Gene Therapy of the Nervous System, Protocols for Neural Cell Culture (2nd ed. S. Fedoroff and A. Richardson Eds., Humana Press, 1997); Ho, Meth. Cell Biol. 43:191-210 (1994); Geller, A. I., et al., Proc. Natl. Acad. Sci. USA 90:7603-7607 (1993); and Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)), each of which is incorporated herein by reference. Additional techniques useful for the construction of amplicons and other DNA constructs may be found in Sambrook et al. Molecular Cloning: A Laboratory Manual, 2nd ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, (1989), incorporated by reference.

In the present context, an amplicon is a plasmid which contains bacterial plasmid sequences which allow prokaryotic amplification, as well as sequences which allow expression and/or replication in eukaryotic hosts, and signals compatible with a viral packaging system, usually a virus-infected cell monolayer. Amplicons are thus designed to mimic the packaging signals of an intact viral genome such that amplicon DNA replaces the viral DNA in nascent viral capsids. Thus, in the case of HSV, because an HSV virion can package 152-154 kb of DNA (the HSV genome), 31-32 copies of a 4.8 kb HSV-based amplicon vector would be packaged per virion.

Generally, packaging is accomplished *ex vivo* and requires a defective "helper" virus to generate the packaging machinery and capsids. Unfortunately, an inherent

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failing of existing packaging systems is the production of infectious amplicon particles contaminated with helper virus. Consequently, therapies based on standard amplicon/helper virus systems increase the viral load on the host, and may contribute to cytopathic effects. Although techniques such as using conditionally defective viral mutants are designed to reduce cytopathic effects, it is ultimately desirable to avoid exposing the host to any possible additional pathogen.

In a preferred embodiment of the present invention, the amplicons can function without helper viruses and are applied to the site of an active viral infection as unpackaged DNA. Because the amplicon contains signals which mimic those of the actively replicating virus, the exogenous DNA will be packaged into infectious particles *in situ*. Because *in situ* packaging depends on on-going viral replication, the amplicons are only packaged in infected cells. Consequently, the applied DNA is effectively targeted to those specific cells. In addition, because virions containing amplicon vector DNA are indistinguishable from virions containing viral genomes, the packaged amplicon vectors will spread the same as infectious viral particles.

Moreover, with respect to neurotropic viruses such as HSV, the active lesion provides a portal for both the virus and the amplicon to enter the nervous system. Again, because the packaged amplicon is functionally indistinguishable from the packaged virus, it will be transported to the same or similar sites in the peripheral and central nervous system. Consequently, expression of the DNA of the amplicon in neural tissues will occur specifically in HSV-infected regions, thereby increasing the therapeutic effect and greatly decreasing potential side effects.

This embodiment not only avoids the potential dangers to the host, but simplifies production by eliminating the time, effort and expense of helper virus packaging. In addition, this embodiment provides for targeting to the site of active viral infection, and allows targeting to more remote sites of viral residence. Moreover, because this method does not require storage and application of infectious viruses, it provides a more stable and easily formulated medicament.

HSV-based amplicon vectors of the present invention generally contain the following six elements:

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- 1) A backbone vector (for example, pBR322) with a prokaryotic origin of replication, and an antibiotic resistance-conferring gene (usually β -lactamase). This permits amplicon vector replication in bacteria, thereby facilitating amplicon isolation and purification.
- 2) The HSV-1 or HSV-2 a' sequence.
- 3) An HSV-1 or HSV-2 replication origin (either ori_s or ori_L).
- 4) At least one promoter compatible with mammalian cells driving expression of a transgene.
- 5) A transgene.
- 6) A polyadenylation site.

The a' and ori sequences are necessary for the replication and packaging of HSV-based amplicons. The a' sequence serves two functions: HSV replicates by a rolling circle mechanism which produces concatamers of the viral genome; a' provides both the site for cleavage into single genome lengths, and the packaging signal for inserting the unit length DNAs into preformed capsids. The HSV genome consists of two covalently linked components designated L (long) and S (short), each of which contains a corresponding origin of viral replication (ori_s and ori_L , respectively).

Because the a' and ori signals may be poorly recognized by non-type viruses, it is preferred that HSV-1-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-1, whereas HSV-2-based amplicon vectors contain an a' sequence and ori_s sequence derived from HSV-2. Although in the present examples, HSV ori_s sequences provides an origin of replication, allowing amplicon vector DNA replication in HSV-infected cells, an ori_L sequence may be substituted. Moreover, as recognized by one of ordinary skill, analogous sequences from other viruses may be substituted to affect packaging into actively replicating non-HSV virions and to treat diseases other than HSV-1 and HSV-2.

HSV-based amplicon vectors can be designed with a variety of promoters for constitutive or induced expression of any potential antiviral protein. Preferred promoters fall into two, potentially overlapping, classes: those that will direct expression of the transgene in epithelial cells and those which are active in neuronal cells.

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Given the multiple stages of HSV infection, it is preferred that the anti-viral transgene is constitutively expressed in epithelial cells. Consequently, transgene expression from this promoter will coincide with active infection and diminish as the virus is cleared from the epithelial cells. Transgene expression in the lesion will reduce the severity and duration of an infection, promote healing, and induce the virus to enter latency.

In contrast, to limit adverse neurological consequences, it is preferred that expression in neuronal cells is inducible. Ideally, expression in neuronal cells is stimulated by the same signals which stimulate neuronal HSV to break latency and begin replicating. The amplicon will tend to remain in neurons in a 'latent' state similar to HSV, nevertheless, the amplicon-driven transgene will be expressed at the same time that HSV is replicating in the nervous system. Consequently, expression of antiviral compounds in neuronal cells will tend to preserve latency and limit the frequency and/or severity of recurrent herpetic outbreaks.

Thus, the preferred embodiments of the invention comprise DNA-based therapies for HSV infections that will be effective during symptomatic and asymptomatic episodes. Most notably, the inducible neuronal expression of anti-viral proteins will maintain HSV in a virtual latent state. Although expression may be regulated, for example, by using a tetracycline-responsive promoter (Fotaki et al., *Gene Therapy* 4:901-08 (1997)), it is preferred that the neuronal expression is stress inducible. Any stress-inducible host or viral promoter may be used including, but not limited to, promoters for glucose regulated proteins (grps), inducible heat shock proteins such as *hsc70*, *hsp70* and *hsp90*, *c-jun*, *c-fos*. In a preferred embodiment, the *c-fos* gene promoter, which is activated during stress and primary HSV-1 infections, is used to drive inducible neuronal expression of anti-viral proteins. (Gieroba et al., *Brain Res.* 675:329-32 (1995); Senba et al., *Neurosci. Res.* 29:183-207 (1997); Sharp et al., *Neurotoxicol.* 15:51-59 (1994); and Tyrell, *EXS.* 77:255-71 (1996)).

The amplicons of the invention are programmed to express anti-viral compounds such as cytokines, preferably interferons, and more preferably, interferon- γ (IFN- γ). Other useful anti-viral compounds which may be expressed alone or in combination with

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themselves, and/or with IFN- γ , include IFN- α , IFN- β , TNF- α , TNF- β , TGF- α , and anti-viral antibodies. (Wong et al., Nature 323:819-822 (1986); Lu et al., J. Invest. Dermatol. 108:803-808 (1997); Kumano et al., Antiviral Res. 7:289-301 (1987); and Yamada et al., Arch. Virol. 99:101-109 (1988)).

One highly preferred embodiment comprises a HSV-1 or HSV-2-based amplicon containing: 1) a constitutive CMV immediate early gene promoter driving IFN- γ expression; and 2) a second HSV-based amplicon of the same HSV type, containing the stress-inducible c-fos gene promoter driving IFN- γ expression. Alternatively, both the constitutive and stress- promoters may be constructed on a single HSV-1 or HSV-2-based amplicon.

In this highly preferred embodiment, inoculation at the site of viral replication initiates a four tier anti-viral therapy:

First, IFN- γ expression from the CMV promoter exerts an antiviral effect at the site of active infection. Increasing IFN- γ levels repress the CMV promoter reducing amplicon-directed IFN- γ production in the epithelial cells. However, because IFN- γ protein has a half-life of 48 hours, it continues to exert an antiviral effect for the duration of the outbreak.

Second, the replication machinery of the resident virus packages the amplicon in HSV virions *in situ*. Each virion that packages amplicon DNA becomes replication-deficient and reduces the viral load on the host. Moreover, the infectious titer at the mucosal membrane is decreased, thereby reducing the likelihood of transmission.

Third, HSV virions containing amplicon DNA are transported into the nervous system. The expression of IFN- γ in the nervous system expedites HSV replication termination and establishment of latency.

Fourth, as the host is exposed to emotional or physical stress (sunlight, fever, immunosuppression, etc.), cellular events occur that cause the latent HSV to reactivate and begin to replicate. The same stressors that reactivate latent HSV will activate the c-fos gene promoter in the "latent" HSV-based amplicon vector. The activated c-fos promoter will drive IFN- γ production, which will rapidly terminate the HSV DNA replication cycle. Consequently, no HSV particles will be made, and HSV will quickly

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return to a latent state. This will potentially keep HSV in a virtual latent state and block asymptomatic shedding of the virus.

The amplicon DNAs of the present invention may be applied to any stage of an active, primary or recurrent, episode of herpes labialis or genitalis, including vesicular (papule, vesicle, and pustule) and crusting stages. However, it is preferred that the DNA be applied early in the virulence cycle during prodrome (throbbing-tingling) and/or erythema (macule) stages. Although topical application is preferred, the amplicon may be administered by any method including subcutaneously, intradermally, intravenously, intraperitoneally, by inhalation or lavage, orally, mucosally, intranasally, or by targeted injection into a lesion or affected body site, including dermal and mucosal tissues, eye, brain, ganglia and other neural tissues.

The DNA-containing compositions of the invention may be applied alone, complexed, suspended, or dissolved in a carrier. An efficacious amount of a DNA-containing composition and carrier comprises a preparation for the treatment of a viral disease or condition. Acceptable liquid carriers or carrier components include: water, polyethylene glycol, dimethyl sulfoxide (DMSO), oils, (including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil), or mixtures thereof, alone or in combination. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., Mack Pub., Easton, Pa., 1990), incorporated by reference. Carriers can be in the form of mists, sprays, powders, waxes, cremes, salves, ointments, patches, poultices, films, or cosmetic preparations. The DNA composition may also include topical anesthetics such as lidocaine, xylocaine and the like, flavorants, emulsifiers, antibiotics, and known or suspected anti-viral compounds including, but not limited to, idoxuridine, interferons, caffeine, anti-viral antibodies, glutaraldehyde, trifluorothymidine (TFT), nonoxynol-9, acyclovir, penciclovir, famciclovir, valaciclovir, cidofovir, brivudin, sorivudine, n-Docosanol, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine (HPMPC), arildone, penciclovir, ribonucleotide reductase inhibitors, SP-303 from *Croton iechler*, Foscarnet, tromantadine, or 2-(N,N-diallylaminomethylene)-9[[2-hydroxy-1-(hydroxymethyl)-ethoxy]methyl]guanine (U.S.

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Patent No. 4,461,757), tumour necrosis factors alpha and/or beta, ascorbic acid, povidone-iodine, and the like.

Because the amplicon DNAs of the invention are preferably administered topically, it is preferred that the DNA is complexed with, dissolved, or suspended in a delivery vehicle that both facilitates DNA transfer through the stratum corneum to the living cells of the lower epidermis, and promotes the uptake of the DNA into the cells. Since 1966, DMSO, an absorption-enhancing agent, has been investigated as a topical delivery system for nucleoside analogs (Becker, Y., *Virus Genes* 10:217-226 (1995)). In Europe, idoxuridine (IDU) in DMSO (80%) is currently in use for the treatment of herpes labialis, genitalis, and zoster. PEI is a cationic polymer that acts as a DNA "sponge" and efficiently delivers DNA into cells. PEI and similar polycationic polymers can be used to transfect DNA into cells both in vitro and in vivo (Abdallah, B., A., et al., *Human Gene Ther.* 7:1947-1954 (1990); Boletta, A., et al., *Human Gene Ther.* 8:1243-1251 (1997); Boussif, O., et al., *Proc. Natl. Acad. Sci. USA* 92:7297-7301 (1997); Goldman et al., *Nature Biotech.* 15:462-467 (1997)), and has been used to deliver HSV-based amplicon DNA into HSV-infected ocular cells in mice. (Ho, D. Y., *Methods in Cell Biol.* 43:191-210 (1994); Lewandowski, G., et al., *J. Neuroimmunol.* 81:66-75 (1994)). Consequently, preferred carriers comprise DMSO and PEI, alone or in combination.

The invention also relates to the treatment of a patient or organism by administration of an efficacious amount of the DNA compositions described herein. A patient or organism is hereby defined as any person or non-human subject in an experimental model, any person or non-human animal in need of anti-viral therapy, or any subject for whom treatment may be beneficial, including humans and non-human animals. Such non-human animals to be treated include all domesticated and feral vertebrates, preferably but are not limited to mice, rats, fish, including carp, pike, catfish, walleye, turbot and salmon, birds, including ducks, pigeons, chickens, and turkeys, rabbits, hamsters, goats, guinea pigs, deer, donkeys, dogs, cats, swine, horses, cattle, sheep, turtles, lizards, snakes, and non-human primates, including monkeys, macaques, marmosets, chimpanzees, orangutans, gorillas and marmosets.

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Treatment comprises effecting any observable or measurable anti-viral effect in a patient or organism. Observable or measurable anti-viral effects include, but are not limited to, reducing viral titer; decreasing viral shedding and infectivity; preventing, limiting, or retarding the spread of the virus in an infected organism; eliminating or reducing the severity and/or duration of an active viral infection, accelerating healing of virus-induced lesions or conditions, promoting, inducing, maintaining or reinforcing viral latency; reducing mortality rates associated with viral infection; and providing prophylactic protection from viral disease, or the effects of a viral disease, by administering the DNA compositions of the invention to an uninfected patient or organism.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

Example 1

***In situ* packaging of HSV-based amplicon DNA into HSV virions**

To demonstrate that unpackaged DNA can be packaged *in situ* by an active viral infection, we inoculated DNA of the HSV-1-based amplicon vector HSV-1(pIFN- γ) (Figure 1) into the eyes of HSV-2 infected mice. The construction of HSV-1(pIFN- γ) and the intraocular model of infection are described in detail in Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998) and Lewandowski, G., et al., J. Neuroimmunol. 55:23-34 (1994), respectively. Briefly, the vitreous compartments of 25 BALB.cByJ mice (6-8 weeks of age) were infected with HSV-2(G) (2×10^4 pfu = $2300 \times \text{LD}_{50}$). HSV-1(pIFN- γ) DNA was administered intraocularly to ten mice at 6h pre-inoculation and to five mice at 16h postinoculation. The inoculi were formulated to contain 3 μg of an HSV-1-based amplicon plasmid expressing IFN- γ (HSV-1(pIFN- γ) mixed with (i) 5% glucose-saline, or (ii) 9 equivalents of polyethylenimine (PEI), a transfection agent, in the same vehicle.

As expected, the ten HSV-2-infected mice treated with vehicle only died by day 5. By contrast, the five HSV-2-infected mice treated postinoculation with either HSV-1(pIFN- γ) DNA formulation survived 9-10 days. Of the ten HSV-2-infected mice treated with HSV-1(pIFN- γ) DNA pre-inoculation, all survived at least 11 days and 33%

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completely recovered. These results indicated that a single administration of HSV-based amplicon plasmid (DNA) expressing IFN- γ could prolong survival of HSV-2-infected mice.

Although the increased survival of HSV-1(pIFN- γ)-treated animals suggests that the amplicon may have been packaged *in situ* by the actively replicating HSV-2 virus, *in vitro* studies (not shown) indicate that HSV-2 virions inefficiently package and replicate amplicon based on HSV-1 DNA. This is most likely due to the mismatch of HSV-2 virions with HSV-1 α' and/or HSV-1 ori_s sequences.

Example 2

HSV-based amplicon DNA is transported into the nervous system where it expresses high levels of IFN- γ

HSV-1(pIFN- γ)-containing virions have previously been demonstrated to be transported from the eye to the brain of HSV-2-infected mice (Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). To demonstrate neuronal transport and expression, HSV-1(pIFN- γ) and HSV-1(pLac) DNAs (Geller and Breakefield, Science 241:1667-79 (1988), were packaged into HSV-1 virions *in vitro* (Ho, D. Y., Methods in Cell Biol. 43:191-210 (1994); Lewandowski, G., et al., J. Neuroimmunol. 81:66-75 (1998)). Mice were then co-inoculated with amplicon DNA and HSV-2 virus by direct injection into the vitreous compartment of the eye. At various times postinfection IFN- γ production was measured in the brain using an ELISA kit (Genzyme Corp., Kendall, MA). An acceptable ELISA assay is also described in Antibodies: A Laboratory Manual, (Harlow & Lane eds., 1988), Cold Spring Harbor Laboratory Press (incorporated by reference).

As shown in Figure 2, very little IFN- γ was detected in the brain during HSV-2-infection (hatched bar). However, high IFN- γ levels were detected in the brains of HSV-2-infected mice co-inoculated with HSV-1(pIFN- γ) (solid bars), strongly suggesting that HSV virions containing HSV-based amplicon vector DNA are transported from the periphery into the nervous system.

Example 3**Experimental models of herpes labialis and herpes genitalis.**

Numerous models of HSV pathogenesis are known in the art and are briefly described in Richard J. Whitley, Herpes Simplex Viruses, Fields Virology, 3d. ed., Ch. 73, each of which may be used in the practice and refinement of this invention. Although the ocular model used in the above demonstrations is useful for studying certain neurotropic effects, it provides only a limited representation of the clinical course of HSV infections. Moreover, rapid destruction of non-replicating retinal cells in the HSV-2 ocular model severely limits HSV-based amplicon DNA cellular uptake and forbids multiple applications of HSV-based amplicon vector DNA. Consequently, this application presents novel experimental models of herpes labialis and herpes genitalis in cotton rats and mice which are particularly useful in describing the invention disclosed herein.

Herpes labialis models were established in inbred male and female Sigmodon hispidus cotton rats and BALB.cByJ mice (6-8 weeks of age). Although both mice and cotton rats are susceptible to herpes labialis, infectious virus titers in the mouse nervous system are higher than those in the rat nervous system. In addition, the process of HSV-1 induced lesion formation and healing in mice requires >15 days, as compared to 10 days in the rat. Consequently, the progression of herpes labialis (shedding, lesion formation, and resolution) in cotton rats provides a better model for clinical human disease.

Although the time course of the herpes labialis in cotton rats closely simulates human condition, the wealth of immunological and neurobiological reagents for mice enable a more detailed examination of changes in the immune response and nervous system after application of the instant constructs. Consequently, information obtained by testing the constructs in both systems may be useful for the design of safe and efficacious human treatments. Such treatments may be analyzed in both clinical studies and in human modes of labial and genital herpes such as those described in Rooney et al., Lancet 338:1419-22 (1991) and Rooney et. al., J. Infect Dis. 166:500-506 (1992), both of which are incorporated by reference.

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Animals are anesthetized by methoxyflurane inhalation and the center of the lower lip is scratched with a 21-gauge needle. The scratched area is swiped with a sterile cotton swab soaked in HSV-1 inoculum (1×10^7 pfu/ml). The animals are then characterized with respect to: (1) lesion formation and healing, (2) infectious virus recovery from lips, trigeminal ganglia and brains, and (3) immunohistochemical detection of HSV antigens in lips, trigeminal ganglia and brains

Example 4

HSV-1 induced herpes labialis in cotton rats and mice

The progression of HSV-1 induced herpes labialis in the cotton rat model is shown in Figure 3. The prodrome and erythema stages occurred at 1d-2d (days postinoculation) (Panel A). Lip lesions were first visible at 2d-3d (Panel B), and progressed to the vesicle stage between 4d and 5d (Panel C). Finally, the lesions began crusting at 5d-6d (Panel D), and healing was nearly complete by 9d-10d (Panels D and E).

HSV-1 titers in lips, trigeminal ganglia and brainstem were determined by standard plaque assay and the results plotted in Figure 4. HSV titers in the lips peaked at 2d (Panel A). The sharp decrease in HSV-1 titers at 3d was indicative of the viral replication termination. The low HSV titers at 5d-7d coincided with viral clearance. These data are in good agreement with the clinical stages of herpes labialis. Specifically, HSV replication and shedding were rapid, and occurred during the first 2-3 days, whereas viral clearance and lesion healing required an additional 7 days.

HSV replication in the trigeminal ganglia began at 2d, peaked at 4d, and declined sharply at 6d (Panel B). In the brainstem, HSV replication occurred through 5d, and was decreased by 6d (Panel C). Infectious HSV was no longer detectable in the trigeminal ganglia and brain at 14d. The HSV recovery time courses suggest that HSV travels from the lip into the trigeminal ganglia, and then from the ganglia into the brain.

Immunohistochemical analysis of excised tissues demonstrates the expression of HSV-1 proteins in infected tissue (Figure 5). HSV proteins are abundant in the lip at 1d (Panel A), and are still detectable at 5d during lesion healing (Panel B). As

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anticipated, at 5d, we detected HSV in the trigeminal ganglia neurons (Panel C), and tract (Panel D). Unexpectedly, we also detected HSV in the brainstem (Panel E) and cerebellum (Panel F).

By 30d HSV establishes a latent infection in the trigeminal ganglia, and brain. The latent infection can be reactivated in cotton rats by immunosuppression with cyclophosphamide (50 mg/kg) and dexamethasone (8 mg/kg). In the cotton rat model, HSV titers were first detected 8 days post-treatment in lips, trigeminal ganglia, and brainstem. (Data not shown).

Similar results were obtained using BALB.cByJ mice. From these data we conclude that HSV inoculation of cotton rats and mice provide excellent models of herpes labialis primary and recurrent infections.

Example 5

Experimental models of HSV-2 induced herpes genitalis

Sigmodon hispidus cotton rats and BALB.cByJ mice are also susceptible to intravaginal HSV-2 inoculation. Female animals (6-10 weeks) are anesthetized by intraperitoneal injections of acepromazine (2.5 mg/kg) and ketamine (25 mg/kg), and intravaginally inoculated with HSV-2(G) (1×10^7 pfu/ml) (20 μ l mice, 75 μ l rats). Adult female mice are susceptible to vaginal HSV-2 during diestrus and pregnancy, but not at estrus. However, with progestin treatment, the animals are continually susceptible to HSV-2. (Baker, D. A. and S. A. Plotkin. 1978). Vaginal infection in mice by herpes simplex type II is enhanced with progesterone. (Proc. Soc. Exp. Biol. Med. 158:131-134; Baker, D. A., et al., et al., Proc. Soc. Exp. Biol. Med. 158:131-134; Parr, M. B., et al.; Lab. Invest. 70:369-380 (1994)). Thus, female cotton rats and mice are injected subcutaneously with 0.01 μ g of estradiol benzoate in peanut oil, followed 24 h later by subcutaneous injection of 2.0 mg of progestin (Depo-Provera, Upjohn, Kalamazoo, MI) in PBS. The animals are inoculated 5 days later.

In the absence of progestin treatment, 40%-60% of HSV-2-inoculated animals became infected. Progestin treatment increased the susceptibility to intravaginal HSV-2 to 100%. We observed 100% morbidity and mortality in the HSV-2-infected animals.

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HSV was recovered from vaginal washes beginning at 1 dpi, and from dorsal root ganglia and spinal cord beginning at 3 dpi. Although the high mortality rate makes this model difficult, it is an excellent paradigm for efficacy testing of anti-herpes therapies.

Example 6

Construction of HSV-2-based amplicon vectors

HSV-2(pCMV)

The HSV-2(pCMV) amplicon vector was constructed as follows: The TK poly(A), neomycin/kanomycin resistance gene (Neo^R), and SV40 origin of replication (SV40 ori) sequences of the pBK-CMV phagemid (Stratagene, LaJolla, CA) (Figure 6), were replaced with the β -lactamase promoter and gene (Amp^R) to generate plasmid pBK-CMV/Amp^R. The HSV-2(G) packaging signal sequence (a' sequence, 550 bp), and origin of replication (ori_s, 667 bp) were amplified from HSV-2(G) genomic DNA (ABI, Gaithersburg, MD) using the polymerase chain reaction (PCR). Each sequence was subcloned into the pCR-Script vector. The HSV-2(G) a' sequence was then cloned into the MluI site of pBK-CMV/Amp^R by blunt-end ligation. Likewise, HSV-2(G) ori_s DNA was cloned into the BspLu11I site, to generate amplicon HSV-2(pCMV) (Figure 7).

HSV-2(pCMV-LacZ)

To remove the bacterial LacZ promoter and a portion of the polylinker, HSV-2(pCMV) will be incubated with endonucleases Sal I and Nhe I. Then the psv- β -galactosidase vector (Promega), which contains the full-length β -gal gene, will be digested with the Hind III endonuclease. The Nhe I and Sal psv- β -gal vector ends will be modified to have blunt ends, and ligated to Nhe I linkers (Stratagene). The modified psv-gal β vector will be digested with Nhe I and Sal I and ligated with the NheI/Sal I-digested HSV-2(pCMV) vector to create the reporter construct HSV-2(pCMV-LacZ) (Figure 8).

HSV-2(pfos-LacZ)

HSV-2(pCMV-LacZ) will be digested with endonucleases Nsi I and Nhe I. The mouse c-fos promoter will be amplified from mouse (BALB.c) brain DNA using PCR. The forward and reverse primers (Life Technologies, Gaithersburg, MD) are engineered such that the amplified c-fos promoter sequence will contain a 5' NsiI site and a 3' NheI

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site. The amplified c-fos promoter will be ligated with the NsiI/NheI digested HSV-2(pCMV-LacZ) amplicon vector, to generate the stress inducible reporter construct HSV-2(pfos-LacZ).

HSV-2(pCMV-mulFN- γ)

The β -gal gene will be removed from the HSV-2(pCMV-LacZ) vector by blunt end digestion with endonucleases Nhe I and Pvu II. The mouse IFN- γ coding sequence will be amplified from the vector pms10 (ATCC, Manassas, VA), using PCR. However, and as understood by those of skill in the art, human IFN- γ sequences could be amplified to provide human therapeutic amplicons. The forward and reverse primers are engineered such that the amplified IFN- γ gene coding sequence will have a 5' Nhe I site and a 3' Dra I blunt end. The amplified mouse IFN- γ will be ligated with the Nhe I-Pvu II digested vector to generate HSV-2(pCMV-mulFN- γ) (Figure 9).

HSV-2(pfos-mulFN γ)

The CMV promoter in HSV-2(pCMV-mulFN- γ) will be replaced with the mouse c-fos promoter, described above, to generate HSV-2(pfos-mulFN γ) (Figure 10).

HSV-2(pCMV-crIFN γ)

The cotton rat (*Sigmodon hispidus*) IFN- γ coding sequence (crIFN- γ) will be amplified using PCR from a cotton rat cDNA library. The forward and reverse primers will be engineered such that the amplified crIFN- γ gene will have a 5' Nhe I site and a Dra I blunt 3' end. As described in above, the β -gal gene will be removed from HSV-2(pCMV-LacZ), but replaced with the crIFN γ gene, to generate HSV-2(pCMV-crIFN γ) (Figure 11).

HSV-2(pfos-crIFN- γ)

The CMV promoter will be removed from the HSV-2(pCMV-crIFN γ) vector, and replaced with the mouse c-fos promoter, described above, to generate the HSV-2(pfos-crIFN- γ) vector.

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Example 7**Construction of HSV-1-based amplicon vectors**

HSV-1-based amplicon vectors are constructed essentially as described for the HSV-2-based amplicon vectors from pBK-CMV/Amp^R except that the HSV-1 a' sequence and ori_s sequences are amplified from HSV-1(F) virion DNA (ABI) and used in lieu of HSV-2 sequences.

Example 8**In vitro testing of amplicon constructs**

The HSV-1 and HSV-2-based amplicon constructs may be transfected into Vero cells and tested for the production of expression products using standard methods. For example, IFN- γ production from HSV-2(pCMV-mulIFN γ) and HSV-2(pfos-mulIFN γ) vector DNA's are measured at 24h using an ELISA kit from Genzyme. Similarly, IFN- γ production from HSV-2(pCMV-crIFN γ) and HSV-2(pfos-crIFN γ) vector DNA's is measured at 24h with an ELISA assay optimized to detect *Sigmodon hispidus* IFN- γ .

Example 9**HSV-2(pCMV) is packaged into HSV-2 virions in vitro**

Vero cells were transfected with HSV-2(pCMV) DNA from 10 clones, and then 24h later infected the cells with HSV-2(G) (0.01 pfu/cell). At 48h postinfection, the culture supernatants were transferred to fresh Vero monolayers. After 24h, total DNA was isolated from the cells and samples were then screened for the β -lactamase gene by PCR. The presence of the β -lactamase gene in all clones indicated that the HSV-2-based amplicon DNA was successfully packaged and replicated in HSV-2(G) virions.

Example 10**Optimizing the administration of HSV-based amplicon vector DNA, and *in situ* packaging into HSV virions.**

The HSV-based amplicon constructs described above contain both the DNA packaging/cleavage signal and the HSV ori_s. Thus, the HSV virions in herpetic lesions will package topically applied HSV-based amplicon DNA. Each HSV virion can package

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152-154 kb of DNA; thus, several copies of an HSV-based amplicon vector could be packaged per virion. Clearly, the level of transgene expression (e.g., β -gal or IFN- γ) will depend on the efficiency of HSV-based amplicon DNA packaging into HSV virions. Moreover, the therapeutic effect of the HSV-based amplicon will depend on the level of transgene expression. Consequently, it is desirable to optimize the conditions for *in situ* packaging in the cotton rat and mouse model systems. This will entail determining: 1) an optimal vehicle for topical DNA delivery; 2) the optimal timing and dosing schedule (e.g., prodrome, erythema, papule, vesicle, pustule, crusting, or scab, single vs multiple applications); and 3) the amount in μ g of HSV-based amplicon vector DNA that is most efficiently packaged.

The results obtained using the model systems can then be extrapolated to human patients and used to plan safety and efficacy studies for the topical treatment of herpes labialis and genitalis and related conditions.

Vehicles for DNA delivery

The following experiments provide guidelines for testing the efficacy of DMSO and PEI carriers using the HSV-1-based system. The same basic experiment will be done with the HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, (without virus) and may be modified to test the efficacy of any other formulation.

Topical administration of HSV-based amplicon DNA in DMSO

To determine if DMSO will enhance the absorption and penetration of topical HSV-based amplicon DNA, HSV-1(pCMV-LacZ) and HSV-2(pCMV-LacZ) DNA in various carriers will be applied to uninfected animals with intact lips. Each application will contain a high concentration (5 μ g) of amplicon DNA in order to increase the signal for low degrees of penetration.

Animals will be anesthetized by intraperitoneal injections of ketamine (25 mg/kg) and acepromazine maleate (2.5 mg/kg). 5 μ l of the following formulations will then be applied to a small area in the center of the lower lips of cotton rats and mice.

Group 1: DMSO (80%)/saline (20%);

Groups 2 & 6: HSV-1(pCMV-LacZ) DNA (2), and HSV-2(pCMV-LacZ) DNA (6) in saline;

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Groups 3-5: HSV-1(pCMV-LacZ) DNA in (3) DMSO (80%)/saline (20%); (4) DMSO (40%)/saline (60%); (5) DMSO (10%)/saline (90%);

Groups 7-9: HSV-2(pCMV-LacZ) DNA in (7) DMSO (80%)/saline (20%); (8) DMSO (40%)/saline (60%), and (9) DMSO (10%)/saline (90%).

10 animals in each group will be analyzed at 0.5, 2, 6, 24, and 48 hours post-treatment. Animals will be sacrificed by CO₂ inhalation, and the lower lips removed. The excised lip tissues will be bisected and fixed in a 0.005% glutaraldehyde/2% paraformaldehyde solution for 1 hour at 4°C. After washing in PBS, the tissues will be placed in a β -gal staining solution (35 mM ferricyanide, 35 mM ferrocyanide, 2 mM MgCl₂, 0.02% Tween, 0.02% Nonidet P-40, 1mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactosidase) overnight at 37°C. After the tissues are washed with PBS, blue staining in the tissue will indicate the β -gal expression from the HSV(pCMV-LacZ) constructs. The lateral and ventral distances between the farthest blue staining and the application site will be measured. These values will be used to determine the diffusion/penetration rates and distances corresponding to each formulation. The ANOVA statistical test will be used to determine whether any significant differences occur between formulations. 10 μ m cryostat sections from the tissues will be mounted on charged microscope slides and incubated for 30 min. in the β -gal staining solution. β -gal expression and histopathological changes in the tissues will be monitored by light microscopy. An optimal delivery system will be the formulation that significantly increases the penetration distance from the application site.

Topical administration of HSV-based amplicon DNA complexed with PEI

An increase in cellular uptake will increase the amount of amplicon DNA available to be packaged by HSV virions. The amplicon DNA (5 μ g) will be mixed with various PEI equivalents (PEI equivalents are based on the nitrogen content of the polymer) ten minutes before application to allow DNA-PEI complex formation. A small scratch centered on the lower lips of anesthetized animals will be made with a 21-gauge needle. The following DNA-PEI formulations will be applied to the scratched areas.

Groups 1 & 6: HSV-1(pCMV-LacZ) (1), and HSV-2(pCMV-LacZ) (6) in saline;

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Groups 2-5: HSV-1(pCMV-LacZ) with: (2) 5 PEI equivalents (equ.), (3) 10 PEI equ., (4) 15 P, EI equ., (5): 20 PEI equ.;

Groups 7-10: HSV-2(pCMV-LacZ) with: (7) 5 PEI equ., (8) 10 PEI equ., (9) 15 PEI equ., (10) 20 PEI equ.

Ten animals will be evaluated per group at 24, 48, and 72 hours post-application. Animals will be sacrificed by CO₂ inhalation, and the lower lips will be removed. β -gal expression will be used as an indication of HSV-based amplicon DNA uptake and expression. Five dissected lips per group will be processed for β -gal expression, and prepared for microscopic analysis as described above. Using a light microscope equipped with a 35-mm camera we will obtain photographs and count the blue-stained cells in each sample. β -gal activity will be quantified in the other 5 dissected lips per group. A 10% homogenate (weight/vol.) will be prepared from the dissected lips in phosphate buffer (pH 7.4). After 2 freeze-thaw cycles, cellular debris will be removed by centrifugation. β -gal activity in the resulting sample supernatants will be measured using a colorimetric assay kit (Promega). Sample proteins will be measured using the BioRad assay and β -gal activities calculated as units per mg protein. Data from cell counts and β -gal activity assays will be analyzed using the ANOVA statistical test. Data from groups 1 and 6 will establish the baseline values for cellular uptake of amplicon DNA without vehicle. Cellular uptake values obtain from groups 2-5, and 7-9 will be compared to the baseline values, and will indicate whether PEI offers any improvement over the use saline alone.

Topical administration of HSV-based amplicon DNA complexed with PEI

Once the optimal (Opt.) concentrations of DMSO and PEI carriers have been determined from the above experiments, uninfected cotton rats and mice with intact lips will be tested to determine whether DMSO can facilitate the penetration and cellular uptake of DNA-PEI complexes, without artificially disrupting the lip surface. 5 ug of DNA will be applied to the lower lip of anesthetized animals with a soft applicator brush. The following formulations will be tested. Group 1: DMSO (Opt.)/PEI (Opt.)/saline;

Groups 2-5: HSV-1(pCMV-LacZ) in (2) DMSO (Opt.)/PEI (Opt.)/saline; (3) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (4) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (5)

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DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 6-9: HSV-2(pCMV-LacZ) in (6) DMSO (Opt.)/PEI (Opt.)/saline; (7) DMSO (0.5 Opt.)/PEI (Opt.)/saline; (8) DMSO (0.5 Opt.)/PEI (0.5 Opt.)/saline; (9) DMSO (Opt.)/PEI (0.5 Opt.)/saline; Groups 10-11: HSV-1(pCMV-LacZ) in (10) DMSO (Opt.)/ saline; (11) PEI (Opt.)/saline; Groups 12-13: HSV-2(pCMV-LacZ) in (12) DMSO (Opt.)/ saline; (13) PEI (Opt.)/saline. (0.5 Opt. Refers to one-half of the optimal concentration)

Ten animals will be evaluated per group at 2, 6, 24, 48, and 72 hours post-application. Animals will be sacrificed by CO₂ inhalation, and the lower lips will be removed. Data will be collected on: (a) diffusion/penetration distances, (b) qualitative and quantitative analysis of cellular β -gal expression, and (c) β -gal activity levels in tissue homogenates.

Five animals per group will be used to obtain the diffusion/penetration distances and cellular uptake data, and 5 animals will be used for β -gal activity assays. Once again, the penetration distances will indicate the ability of the vehicle to deliver the HSV-based amplicon DNA through the stratum corneum to the living cells. β -gal expression will indicate the ability of the vehicle to enhance cellular uptake of the HSV-based amplicon DNA. Histological data from group 1 will establish whether the vehicle causes severe skin irritation or histopathology. Data from groups 10-13 will establish the baseline values for DMSO and PEI formulations alone. Data obtained from groups 2-9 will be compared to the baseline values and will indicate whether the combination of DMSO and PEI offers any improvement over the use of either vehicle alone. The ANOVA statistical test will be used to determine if any significant differences occur between the formulations.

The basic experiments described above are for the HSV-1(pCMV-LacZ), and HSV-2(pCMV-LacZ) constructs in the labialis cotton rat and mouse models, without HSV. The results of these experiments will be uncomplicated by the presence of herpetic lesion. However, the presence of a herpetic prodrome or lesion may change the requirements of the vehicle formulation. Also, the topical delivery vehicle formulation may differ somewhat for herpes genitalis. Accordingly, the same basic experiments will be done with the following variations: (1) HSV-1(pCMV-LacZ) construct with HSV-1(F)

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in the labialis models, (2) HSV-2(pCMV-LacZ) construct in the HSV-2(G) genitalis models, without virus, and (3) HSV-2(pCMV-LacZ) construct with HSV-2(G) in the genitalis models.

Optimization of dosing schedule of HSV-based DNA amplicon for *in situ* packaging.

Herpes labialis and genitalis lesions occur in 8 stages. Infectious virus is present from the prodromal stage through the pustule (late vesicular) stage. However, current topical treatments of HSV-1 and HSV-2 infections administered after early prodromal or macule (erythema) stages are relatively ineffective (Hamuy, R., et al., Eur. J. Dermatol. 8:310-319 (1998); Spruance, S. L., In E. DeClercq (ed.), Martinus Nijhoff Publishing, p. 69-86 (1998); Spruance, S. et al., J. Infect. Dis. 161:191-197 (1990)). Because the HSV-based amplicon DNA therapy of the present invention preferably incorporates *in situ* HSV-based amplicon DNA packaging by HSV virions, the instant therapy may be effective from the prodromal stage through the vesicular stage.

To determine an optimal dosing schedule, 5 µg of HSV-1(pCMV-LacZ) DNA will be prepared in an optimal delivery vehicle and administered at various times pre- and post-HSV-1(F) inoculation. The HSV-1(pCMV-LacZ) in vehicle will be applied to a small area in the lower lip center of anesthetized animals at the following times.

Groups 1-3: (1) 16h pre-inoculation, (2) 6h pre-inoculation, and (3) 2h pre-inoculation. Pre-inoculation applications will allow cellular uptake of the HSV-1(pCMV-LacZ) DNA before the virus is introduced. This is similar to the HSV-based amplicon DNA packaging protocols used in tissue culture.

Groups 4-5: (4) 2h post-inoculation, and (5) 12h post-inoculation. These groups will represent the prodrome stage of herpes labialis; HSV is present and beginning to replicate, but a lesion is not observed.

Groups 6-10: (6) 24h post-inoculation, (7) 48h post-inoculation, (8) 72h post-inoculation, (9) 96h post-inoculation, and (10) 120h post-inoculation. These groups will represent the progressive stages of herpes labialis.

Group 11: 2h pro-mock inoculation. This group will serve as the negative control for *in situ* packaging by HSV virions.

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Groups 1-3, & 11 will be sacrificed 48h after inoculation. Groups 4-10 will be sacrificed 48h after amplicon DNA application. The lips will be removed and tissue homogenates (10% wt./vol.) will be prepared in RPMI 1640 with 2% FBS, as described above. All of the sample supernatants will contain HSV-1 virions with infectious DNA. The supernatants will also contain HSV-1 virions with amplicon DNA, if *in situ* packaging of the HSV-1(pCMV-LacZ) DNA has occurred. We will plate the sample supernatants onto confluent Vero cell monolayers in 96 well plates in triplicate. The plates will be placed at 37°C for 48-72 hours, and then processed for β -gal activities and protein concentrations. Because of the large number of samples to process, data collection will be facilitated by use of a microtiter plate reader set at a wavelength of 420 nm. β -gal activities for each group will be compared and analyzed using the ANOVA statistical test.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. As recognized one of skill in the art, the same basic tests may be done with any number of variations including: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Optimizing the amount of HSV-based amplicon DNA amount for *in situ* packaging

Ideally, the absolute amount HSV-based amplicon DNA that can be packaged *in situ* depends on the DNA to HSV virion ratio. In accord with the results of the preceding experiments, the following amounts of HSV-1(pCMV-LacZ) DNA prepared in the optimal delivery vehicle will be applied to cotton rats and mice.

Group 1: 0.0 μ g;

Groups 2-7: (2) 0.5 μ g; (3) 1.0 μ g; (4) 5.0 μ g; (5) 10 μ g; (6) 50 μ g; (7) 100 μ g;

Groups 8-13: The same as groups 2-7, except that HSV-1(pCMV-LacZ) DNA will be applied at a different time post-HSV inoculation.

Ten animals per group will be sacrificed at a predetermined time point post HSV-1(pCMV-LacZ) DNA application. The lips will be removed and tissue homogenates prepared. The supernatants will be analyzed for the presence of HSV virions containing HSV1(pCMV-LacZ) DNA on Vero indicator cells. The β -gal activity values will be plotted

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against the HSV-based amplicon DNA doses, and the data will be analyzed for statistical significance using the ANOVA statistical test.

The basic experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations: (1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models, (2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models, (3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models, and (4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

Example 11

Establishing the parameters affecting amplicon packaging, transport, and expression

The greatest therapeutic value of topical HSV-based amplicon DNA for the treatment of herpes involves (1) packaging of HSV-based amplicon DNA into HSV virions at the lesion site, (2) the spread of HSV-based amplicon DNA into the nervous system and 3) transgene expression in neural cells. While IFN- γ may have antiviral actions at the lesion site, the greatest therapeutic potential of IFN- γ is to facilitate the transition from the productive infection to the latent HSV infection in neurons. Accordingly, this experimental series demonstrates that topically applied HSV-base amplicon DNA is packaged *in situ* into HSV virions, then transported along peripheral nerves, and expressed in the nervous system.

The lips of cotton rats and mice will be inoculated with HSV-1(F). HSV-1(pCMV-LacZ) amplicon DNA will then be applied to the lips of Group 1 animals. Group 2 animals will comprise the control. DNA application will use pre-established conditions for optimal *in situ* packaging of HSV-1(pCMV-LacZ) DNA into HSV-1 virions.

The time course of HSV-1(F) spread from the lip to the nervous system has previously been established using immunohistochemical analysis and infectious virus recovery (titration) assays (data not shown). 15 animals from each group will be sacrificed at 4, 5, 6, 7, 14, and 30 days postinfection. At each time point, 5 animals from group 1 and 3 animals from group 2 (controls) will be perfused with 4% para-formaldehyde in phosphate buffer. The trigeminal ganglia and brains will be removed

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and placed in an 18% sucrose/phosphate buffer solution overnight at 4°C. Frozen sections of 5 µm will be made using a cryostat, and melted onto charged microscope slides. The sections will be incubated with monoclonal antibodies (mAb) specific for β-gal (Boehringer Mannheim), and the HSV-1 immediate early gene, ICP4 (ABI). Primary antibody binding will be visualized with secondary antibodies conjugated to either Cy3 (red fluorescence) or Cy2 (green fluorescence). ICP4 positive- (red), β-gal positive- (green) and double positive cells will be visualized using fluorescence and light microscopy. Cells infected by HSV-1 virions with HSV-based amplicon DNA will be positive for only β-gal (green staining). Cells infected by HSV-1 virions with HSV-1 DNA will be positive for only ICP4 (red staining). Finally, cells infected with both types of HSV-1 virions will be positive for both β-gal and ICP4 (red-, green-, and orange staining). With these data we will determine if HSV-1 virions containing HSV-based amplicon DNA and HSV-1 virions containing HSV-1(F) DNA are transported to the same or different cells in the nervous system.

In addition, 10 animals in group 1 and 8 animals in group 2 will be sacrificed at each time point by CO₂ inhalation to remove the trigeminal ganglia and the brainstem-cerebellum region of the brains. β-gal activity levels will be measured in the tissue homogenate supernatants from 5 animals in group 1 and 3 animals in group 2 per time point as described above. Infectious virus titers at each time point will be measured in the tissue homogenate supernatants from the other 5 animals in each group. The titers of infectious HSV-1 will be plotted against time postinfection. With these data we will establish: 1) the time courses for HSV-1 infection and β-gal transgene expression in the nervous system; 2) whether transgene expression is simultaneously turned off with the termination of HSV-1(F) DNA replication, and; 3) if the time course of HSV infection in the nervous system is altered by the presence of HSV-1 virions with amplicon DNA.

The experiment and analysis described above is for HSV-1(pCMV-LacZ) with HSV-1(F) in the labialis cotton rat and mouse models. The same basic experiment will be done with the following variations:

- 1) HSV-1(pfos-LacZ) with HSV-1(F) in the labialis models;
- 2) HSV-2(pCMV-LacZ) with HSV-2(G) in the labialis models;

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- 3) HSV-2(pCMV-LacZ) with HSV-2(G) in the genitalis models; and
- 4) the HSV-2(pfos-LacZ) construct in the HSV-2(G) genitalis models.

In the herpes genitalis models, the above-described analysis will be done with the lumbosacral dorsal root ganglia, spinal cords, and brainstems. Similar studies will demonstrate transgene (β -gal and IFN- γ) expression during HSV reactivation.

Example 12

HSV-2(pCMV) DNA is packaged *in situ* and transported to neural sites

Eight cotton rats were inoculated with HSV-2 on their bottom lip. Six hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μ l of HSV-2(pCMV) amplicon DNA (5 μ g) mixed with PEI (9 equivalents) was carefully placed on the inoculation site, using a pipettor. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Four rats were sacrificed on day 5, and 4 rats were sacrificed on day 6. A careful examination of the inoculation sites indicated that 6 of the animals showed no signs of lip lesions at the time of sacrifice. The HSV-2(pCMV) vector does not express IFN- γ or any transgene. Thus, the low incidence of lesion formation in this experiment results because HSV-2(pCMV) DNA competed with infectious HSV-2 DNA for packaging. This effectively reduces the titer of infectious HSV-2 and reduces the likelihood of overt lesions.

DNA was isolated from the trigeminal ganglia (peripheral nervous system) and specific brain regions (brainstem & cerebellum) of all animals and tested for the presence of the β -lactamase (ampicillin resistance) gene by PCR. Three of the animals had detectable β -lactamase DNA in brain and/or trigeminal ganglia. Detection of the β -lactamase gene in the brain and trigeminal ganglia of some HSV-2(pCMV)-treated animals indicates that the amplicon DNA can be packaged *in situ* and transported into the nervous system.

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Example 13**Topical application of HSV-2(pCMV) DNA inhibits lesion formation**

Seven cotton rats were inoculated with HSV-2 on their bottom lip. Sixteen hours later, rats were anesthetized and the inoculation site saturated with the delivery vehicle solution (80% DMSO and 5% glucose in saline). Immediately thereafter, 10 μ l of HSV-2(pCMV) amplicon DNA (5 μ g) mixed with PEI (9 equivalents) was carefully placed on the inoculation site of four of the rats. Delivery vehicle and HSV-2(pCMV) vector DNA was topically applied to the inoculation site in this manner twice a day for 5 days.

Lip lesion formation for each rat was photographed daily for 5 days. As expected, by day five, the vehicle-treated rats had large herpetic lesions. In contrast, the amplicon-treated rats did not exhibit any obvious lesions.

Figure 12 illustrates this dramatic reduction in lesion formation. The top panels show two of the HSV-2-inoculated rats treated with vehicle only. The bottom panels show two of the HSV-2 inoculated rats treated topically with HSV-2(pCMV) DNA.

All animals were sacrificed on day 5 and DNA was isolated from neural tissues as above. The β -lactamase gene was not detected in the DNA's from HSV-2(pCMV)-treated animals in this experiment.

This specification is most thoroughly understood in light of the teachings of the references cited herein, all of which are hereby incorporated by reference in their entirety.

The embodiments within the specification provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan recognizes that many other embodiments are encompassed by the claimed invention and that it is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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We claim:

1. **An *in situ* method for packaging DNA into a viral particle comprising applying at least one unpackaged DNA construct to the site of an active viral infection in an organism, wherein at least one applied DNA construct is packaged into viral particles.**
2. The method of claim 1, wherein the viral infection is a Herpesvirus infection.
3. The method of claim 2, wherein at least one DNA construct is an amplicon.
4. The method of claim 3, wherein the amplicon comprises sequences compatible with packaging into an HSV virion.
5. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-1.
6. The method of claim 5, wherein the amplicon further comprises an HSV ori sequence.
7. The method of claim 6, wherein the HSV ori sequence is an HSV-1 ori_s sequence.
8. The method of claim 4, wherein the amplicon comprises a' sequences compatible with packaging into HSV-2.
9. The method of claim 8, wherein the amplicon further comprises an HSV ori sequence.
10. The method of claim 9, wherein the HSV ori sequence is a HSV-2 ori_s sequence.
11. **A method of treating a viral infection comprising the steps of:**
 - 1) **applying a composition comprising at least one unpackaged DNA construct to a site of viral infection in an organism, and**
 - 2) **packaging at least one applied DNA construct *in situ*.**
12. The method of claim 11, wherein the viral infection is a Herpesvirus infection.
13. The method of claim 12, wherein the Herpesvirus is HSV-1 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-1.
14. The method of claim 13, wherein at least one unpackaged DNA further comprises an HSV ori sequence.

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15. The method of claim 12, wherein the Herpesvirus is HSV-2 and at least one unpackaged DNA construct comprises a' sequences compatible with packaging by HSV-2.
16. The method of claim 15, wherein the at least one unpackaged DNA further comprises an HSV ori sequence.
17. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.
18. The method of claim 12, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.
19. The method of claim 11, wherein at least one applied DNA construct is capable of replicating in a host organism in the presence of an active viral infection.
20. The method of claim 11, wherein the composition is applied topically.
21. The method of claim 11, wherein the organism is human.
22. **A method of treating a viral infection comprising the steps of:**
 - 1) applying a composition comprising one or more unpackaged amplicons to a site of viral infection in an organism,**
wherein one or more of the amplicons encode at least one anti-viral compound, and
 - 2) expressing at least one of the encoded anti-viral compound in the organism.**
23. The method of claim 22, wherein the viral infection is a Herpesvirus infection.
24. The method of claim 23, wherein the Herpesvirus is HSV-1.
25. The method of claim 24, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-1.
26. The method of claim 25, wherein one or more amplicons further comprise a HSV ori sequence.
27. The method of claim 26, wherein the HSV ori sequence is a HSV-1 ori_s sequence.
28. The method of claim 23 wherein the Herpesvirus is HSV-2.

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29. The method of claim 28, wherein one or more amplicons comprise a' sequences compatible with packaging by HSV-2.
30. The method of claim 29, wherein one or more amplicons further comprises a HSV ori sequence.
31. The method of claim 30, wherein the HSV ori sequence is a HSV-1 ori_s sequence.
32. The method of claim 22, wherein one or more amplicons are packaged *in situ*.
33. The method of claim 22, wherein at least one anti-viral compound is a cytokine.
34. The method of claim 33, wherein the cytokine is an interferon.
35. The method of claim 34, wherein the cytokine is interferon gamma (IFN- γ).
36. The method of claim 35, wherein the interferon gamma is human interferon gamma.
37. The method of claim 22, wherein at least one antiviral compound is expressed during active infection.
38. The method of claim 22, wherein expression of at least one antiviral compound is constitutive.
39. The method of claim 22, wherein expression of the antiviral compound is stress-inducible.
40. The method of claim 22, wherein at least one antiviral compound is expressed in epithelial cells.
41. The method of claim 22, wherein at least one antiviral compound is expressed in neuronal cells.
42. The method of claim 22, wherein expression of at least one anti-viral compound is directed by a fos promoter.
43. The method of claim 22, wherein expression of at least one anti-viral compound is directed is directed by a CMV immediate early gene promoter.
44. The method of claim 22, wherein the organism is human.
45. The method of claim 22, wherein the organism is a non-human animal.
46. The method of claim 22, wherein the composition is applied topically.

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47. The method claim 23, wherein the infection is an HSV infection and the composition is applied during the prodrome stage.
48. The method claim 23, wherein the infection is an HSV infection and the composition is applied during a vesicular stage.
49. **A composition comprising one or more amplicons capable of directing the inducible expression of at least one anti-viral compound in neuronal cells, wherein the expression of the at least one anti-viral compound in neuronal cells decreases the frequency or severity of HSV reactivation in an organism.**
50. The composition of claim 49, wherein the promoter is stress-inducible.
51. The composition of claim 49, wherein the promoter is a fos promoter.
52. The composition of claim 49, wherein the anti-viral compound is a cytokine.
53. The composition of claim 52, wherein the cytokine is an interferon.
54. The composition of claim 53, wherein the interferon is IFN- γ .
55. The composition of claim 54, wherein the interferon is human IFN- γ .
56. The composition of claim 49, wherein one or more amplicons comprises an HSV a' sequence and an HSV ori sequence.
57. The composition of claim 56, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-1 infection.
58. The composition of claim 57, wherein the at least one amplicon is capable of being packaged *in situ* by an active HSV-2 infection.
59. The composition of claim 57, further comprising a carrier.
60. The composition of claim 49, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.
61. The composition of claim 49, wherein the organism is a human.
62. **A method of reducing the frequency or severity of HSV recurrences in an organism comprising administering the composition of claim 49.**
63. The method of claim 62, wherein the composition is applied topically to the site of an active HSV infection.
64. The method of claim 63, wherein the composition is applied during the prodrome stage.

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65. The method of claim 63, wherein the composition is applied during a vesicular stage.

66. **A composition for treating HSV infections comprising one or more amplicons programmed to express:**

- 1) at least one anti-viral compound in HSV infected epithelial cells, and**
- 2) at least one anti-viral compound in HSV infected neuronal cells, wherein amplicon-directed expression of at least one anti-viral compound in HSV infected neuronal cells is inducible.**

67. The composition of claim 66, wherein at least one anti-viral compound is a cytokine.

68. The composition of claim 67, wherein the cytokine is an interferon.

69. The composition of claim 68, wherein the interferon is IFN- γ .

70. The composition of claim 69, wherein the interferon is human IFN- γ .

71. The composition of claim 66, wherein neuronal expression is stress inducible.

72. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a fos promoter.

73. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by a constitutively active promoter.

74. The composition of claim 66, wherein expression of at least one anti-viral compound is directed by the CMV immediate early gene promoter.

75. The composition of claim 66, wherein one or more amplicons comprise unpackaged DNA.

76. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-1.

77. The composition of claim 76, wherein the one or more amplicons further comprise an HSV ori sequence.

78. The composition of claim 66, wherein one or more amplicons comprises sequences compatible with packaging into HSV-2.

79. The composition of claim 78, wherein the one or more amplicons further comprise an HSV ori sequence.

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- 80. The composition of claim 66, further comprising a carrier.
- 81. The composition of claim 80, wherein the carrier comprises at least one member of the group consisting of DMSO and PEI.
- 82. **A method for treating HSV infection comprising administering the composition of claim 66 to an organism.**
- 83. The method of claim 82, wherein the organism is human.
- 84. The method of claim 82, wherein the organism is a non-human animal.
- 85. The method of claim 82, wherein the composition is applied topically.
- 86. The method claim 82, wherein the composition is applied during the prodrome stage.
- 87. The method claim 82, wherein the composition is applied during a vesicular stage.

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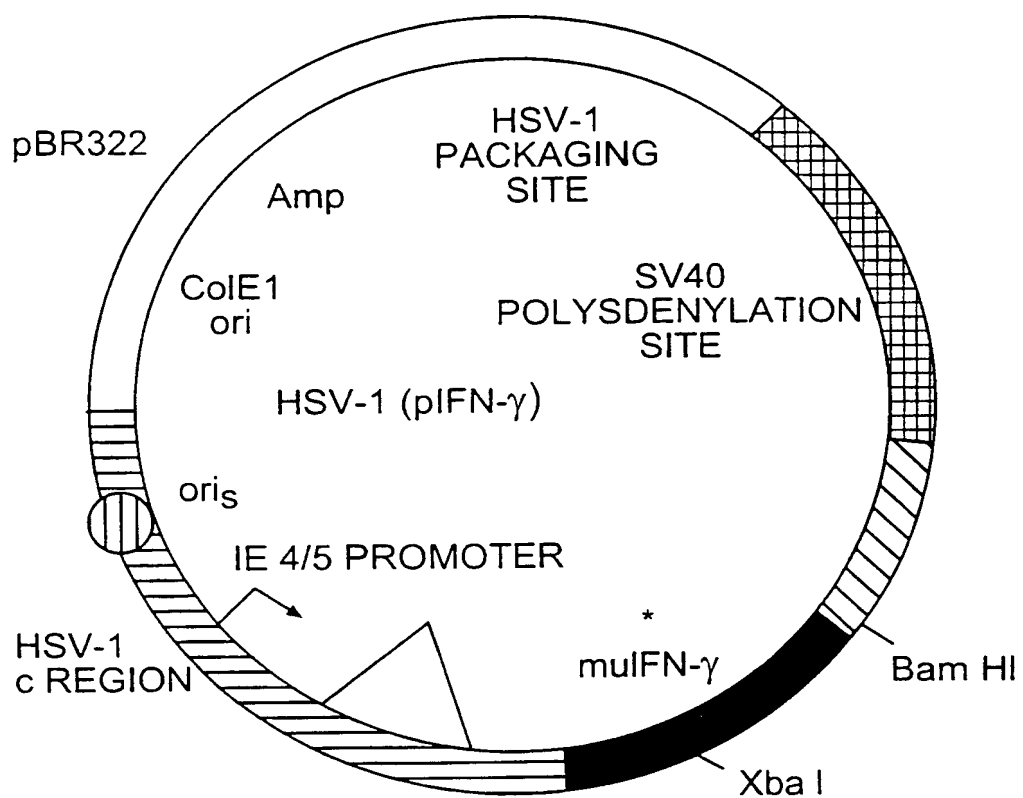
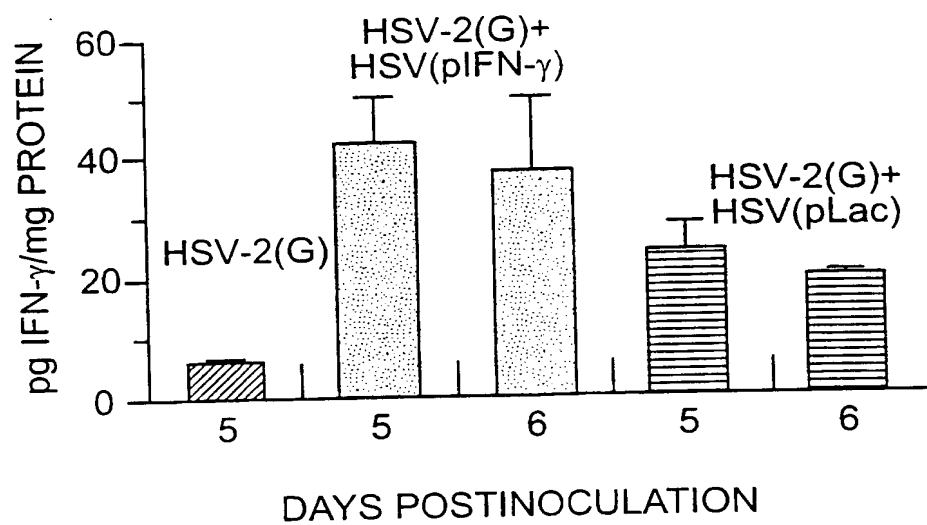


FIG. 1

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**FIG. 2**

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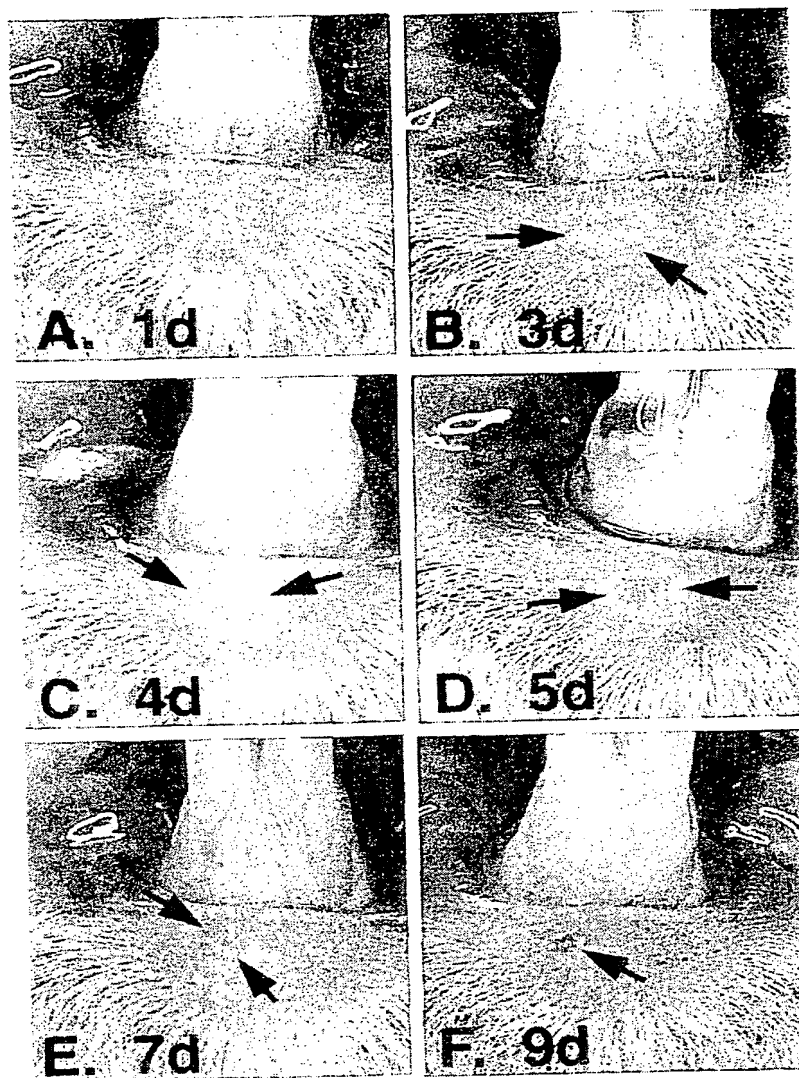
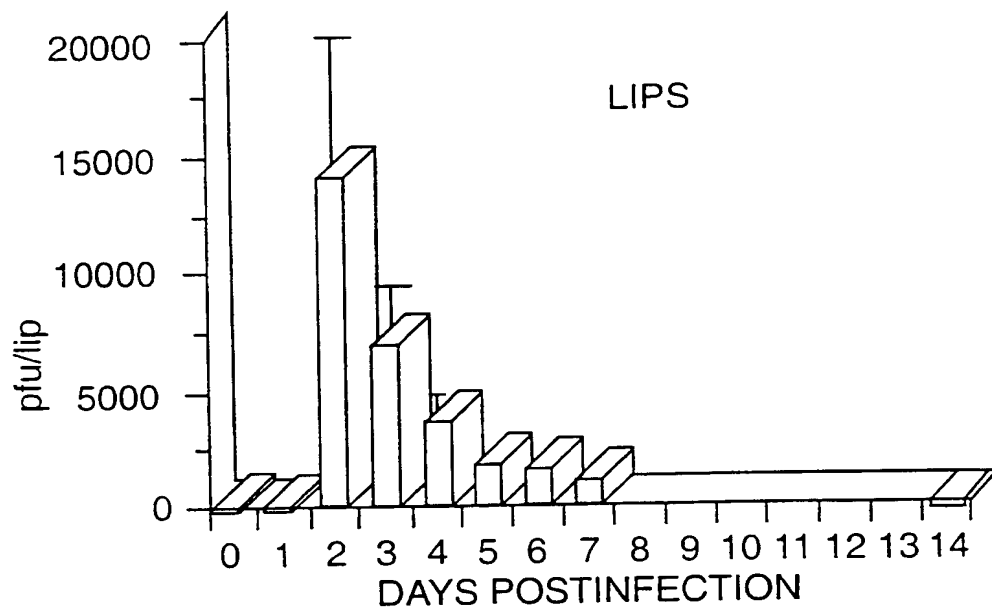
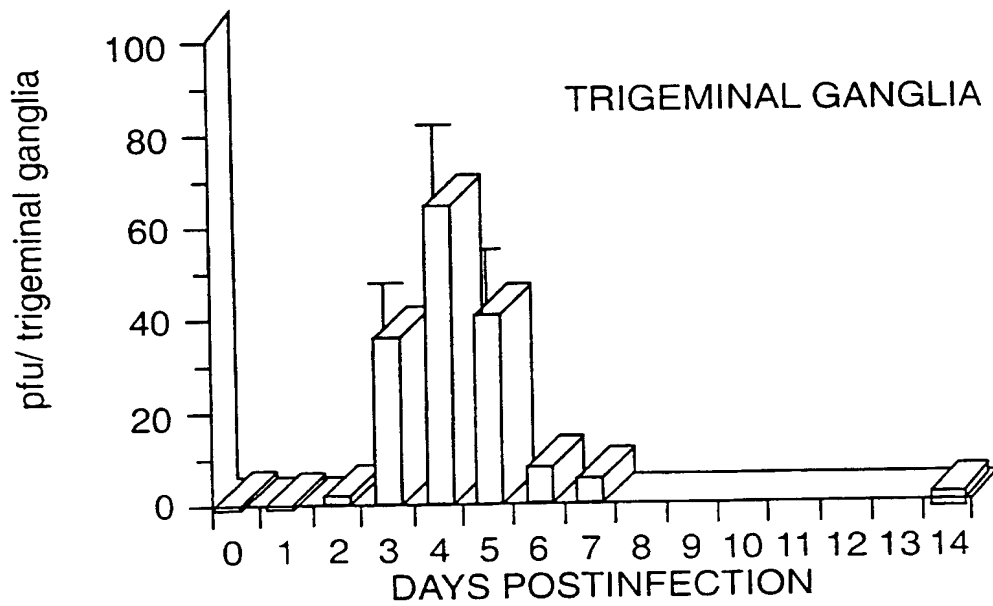
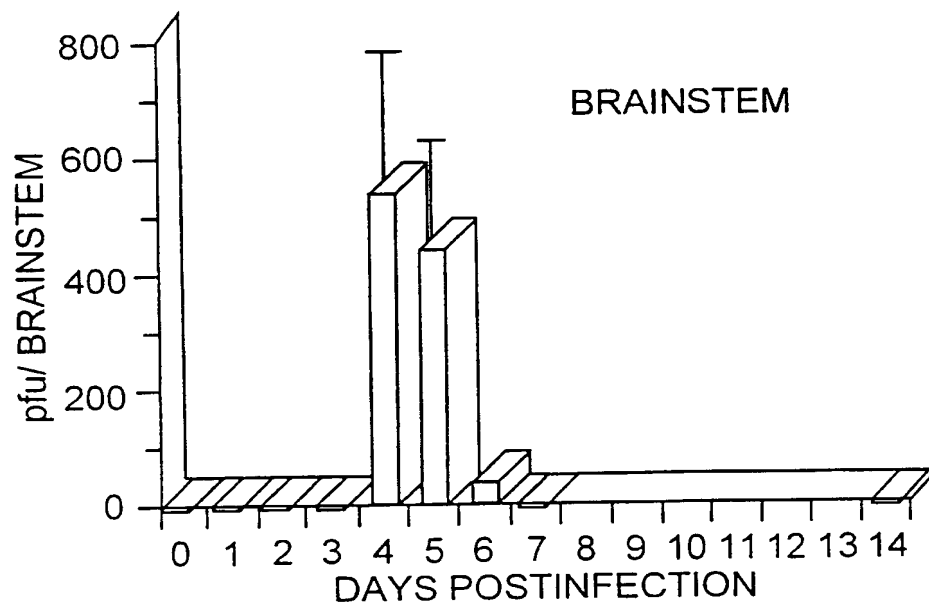


FIG. 3

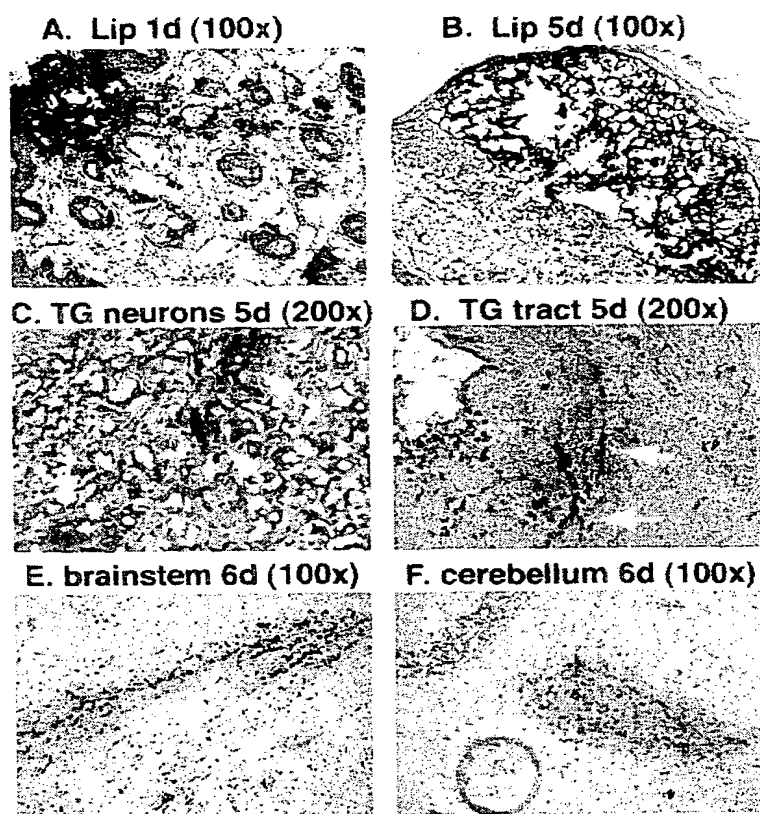
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**FIG. 4A****FIG. 4B**

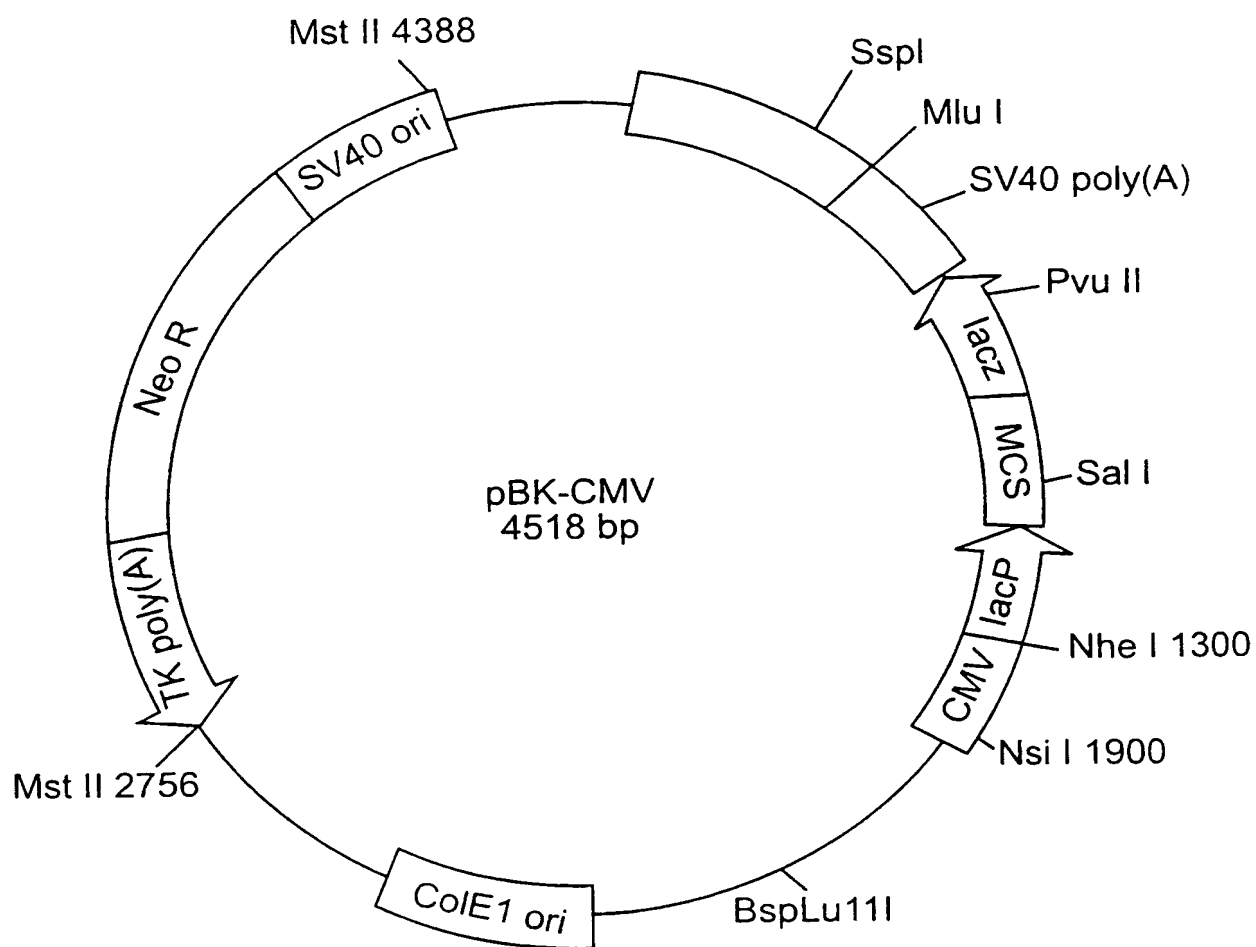
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**FIG. 4C**

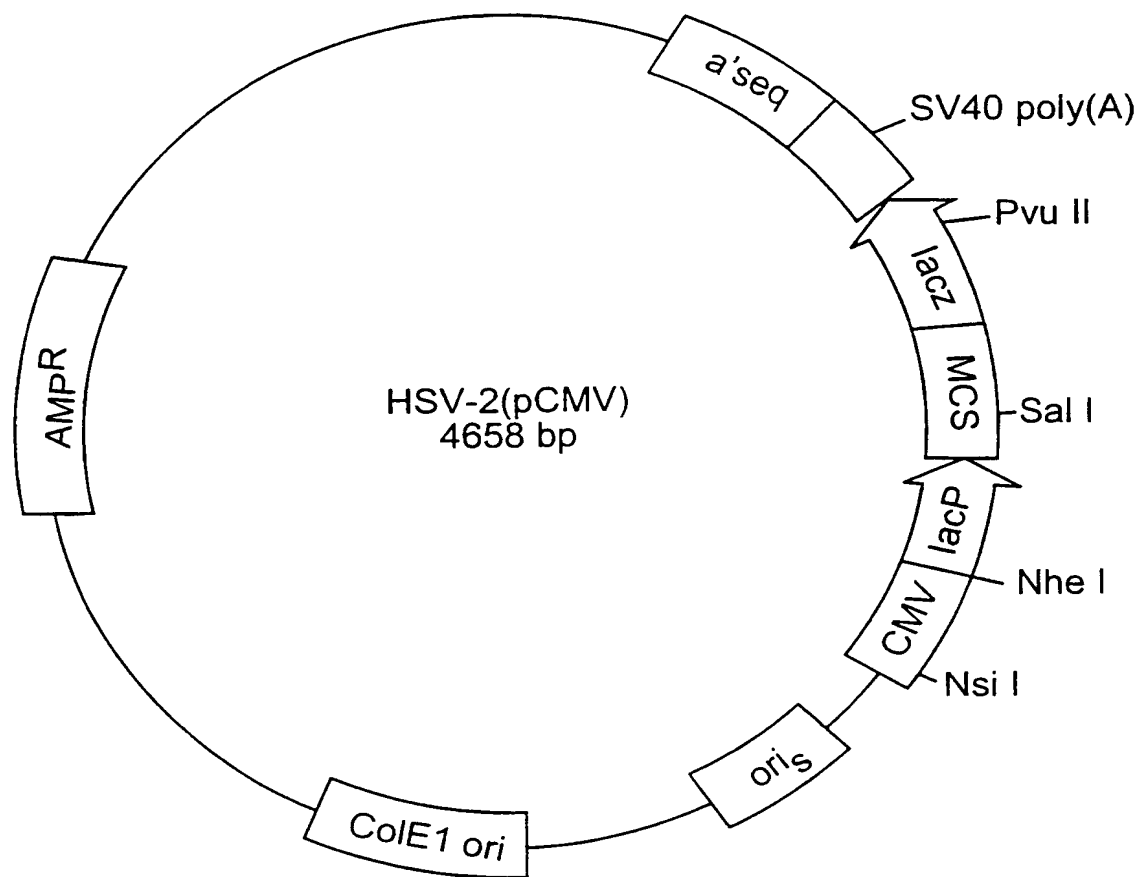
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**FIG. 5**

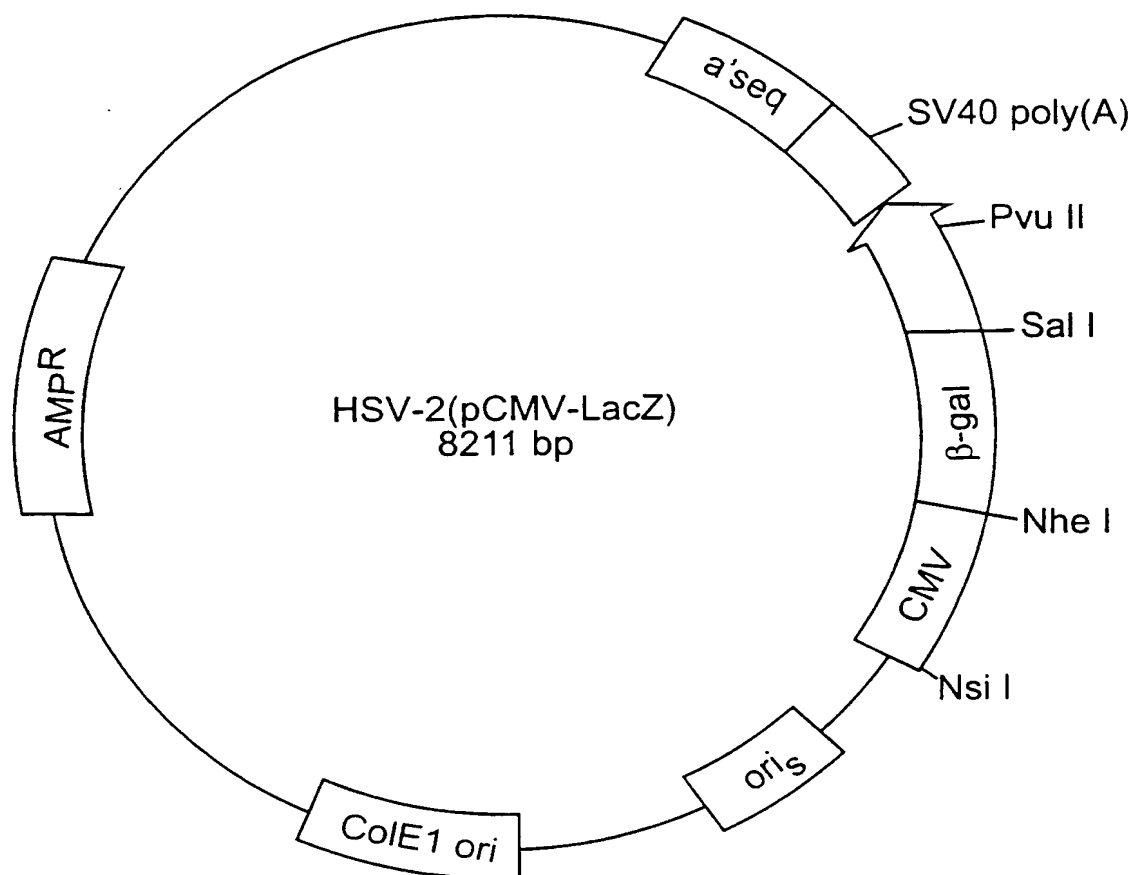
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**FIG. 6**

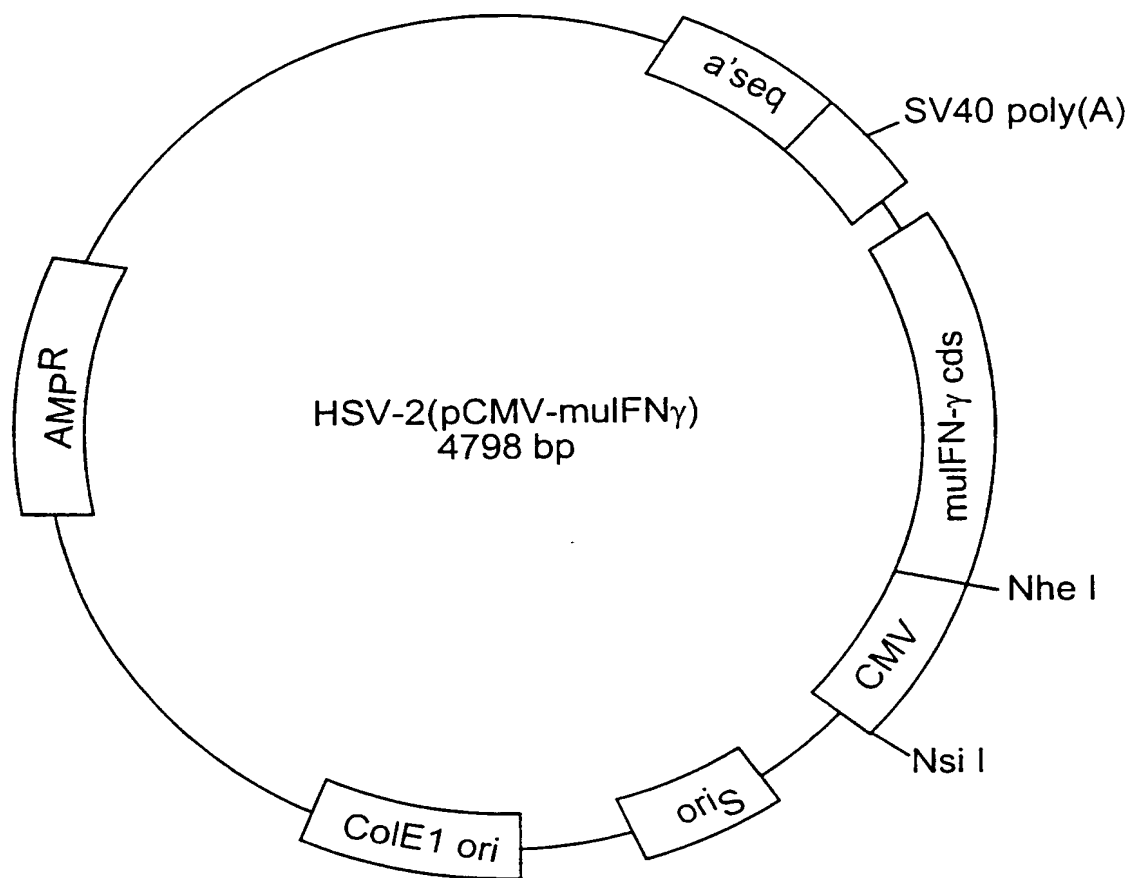
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**FIG. 7**

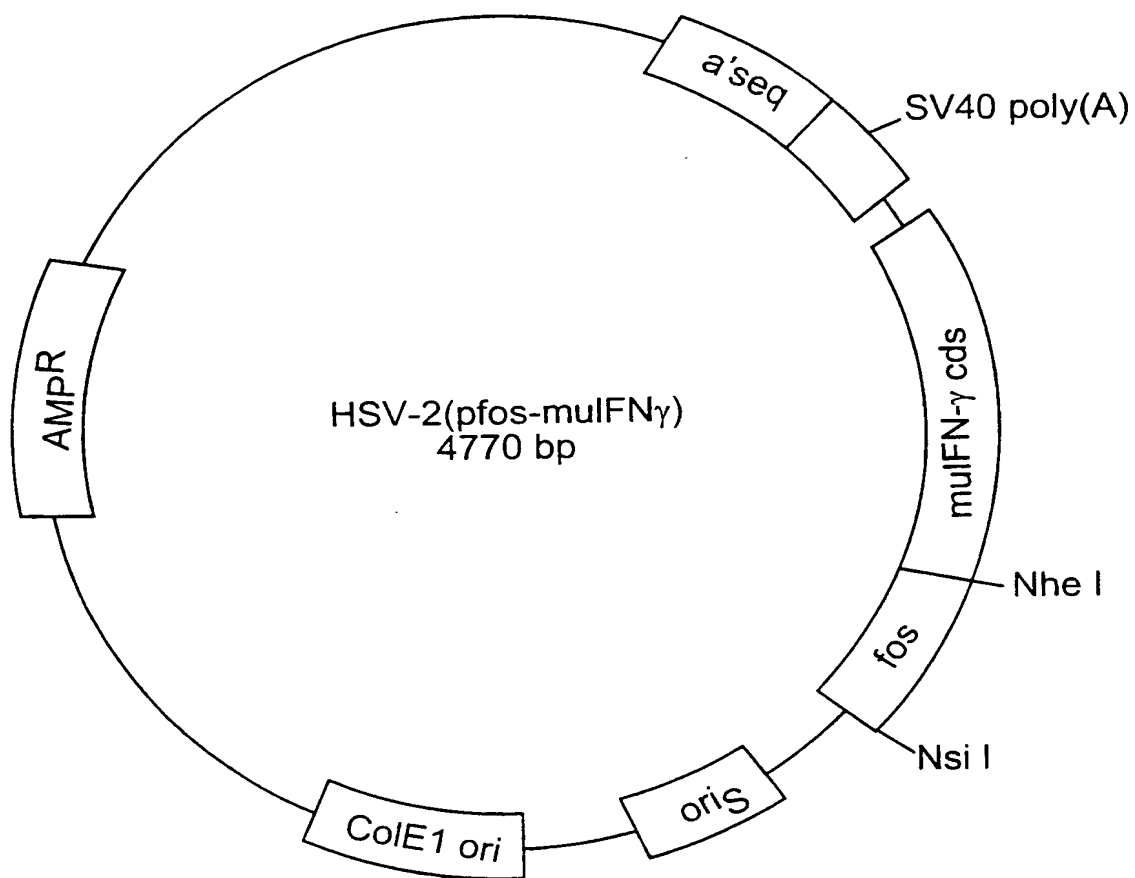
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**FIG. 8**

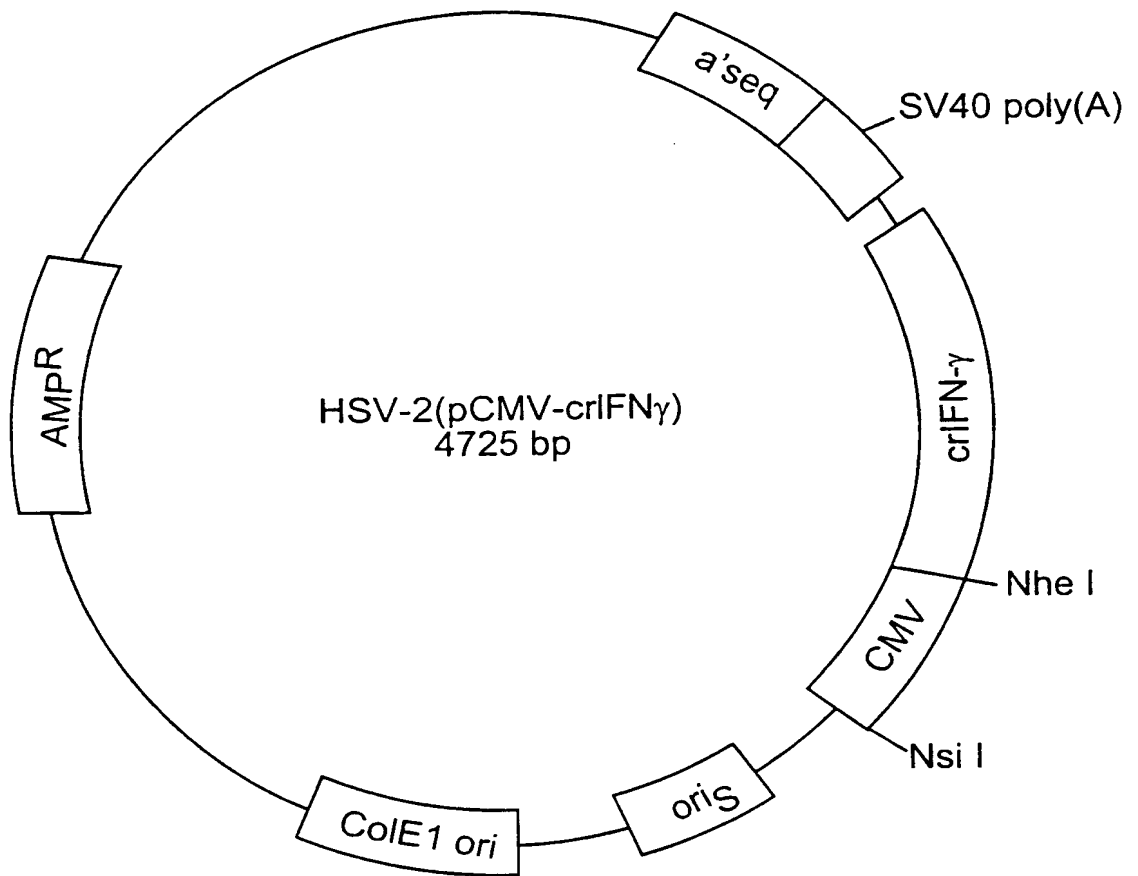
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**FIG. 9**

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**FIG. 10**

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**FIG. 11**

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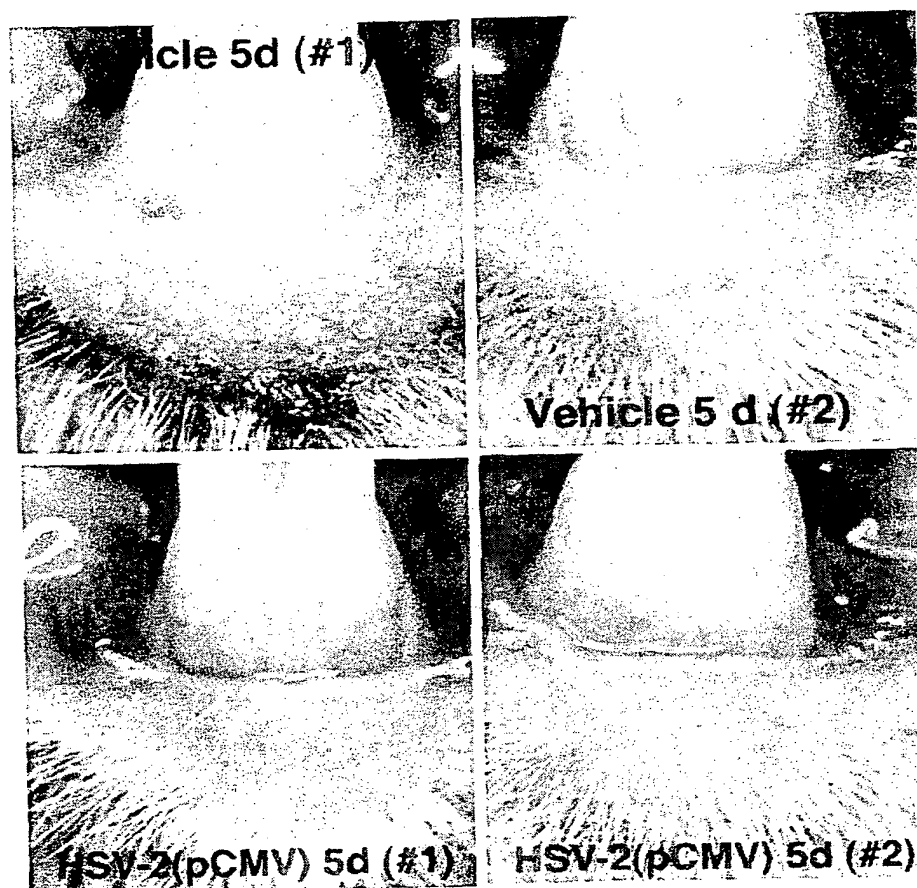


FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05594

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 A61K48/00 A61K38/21 A61K47/20 A61K47/30
A61P31/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, WPI Data, EP0-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	G. LEWANDOWSKI ET AL.: "Evidence that deficient IFN gamma production is a biological basis of herpes simplex virus type 2 neurovirulence" J. NEUROIMMUNOL., vol. 81, 1998, pages 66-75, XP000929623 cited in the application the whole document	49, 52-58, 61, 66-70, 73,76-79
A	B. LU ET AL., : "Topical application of viral vectors for epidermal gene transfer" J. INVEST. DERMATOL., vol. 108, no. 5, 1997, pages 803-808, XP000929624 page 805 -page 808 --- -/--	11-48



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search

4 August 2000

Date of mailing of the international search report

21/08/2000

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Authorized officer

Skelly, J

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/05594

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. STARR ET AL.: "Long-term persistence of defective HSV-1 vectors in the rat brain is demonstrated by reactivation of vector gene expression" GENE THERAPY, vol. 3, 1996, pages 615-623, XP000929634 the whole document -----	
A	WO 99 08713 A (UAB RESEARCH FOUNDATION) 25 February 1999 (1999-02-25) the whole document -----	11-48

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 00/05594

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WO 9908713 A	25-02-1999	AU 8780798 A EP 1015035 A	08-03-1999 05-07-2000
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